CHROMATOGRAPHY '84

Symposia Biologica Hungarica 31

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31

Akadémiai Kiadó, Budapest

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Proceedings of the Advances in Liquid Chromatography
Szeged, Hungary, September 10–14, 1984

Symposia Biologica Hungarica 31

Edited by
H. KALÁSZ and L. S. ETTRE

Authors from Canada, Czechoslovakia, Egypt, the German Democratic Republic, Hungary, Poland, Romania, Sweden, the USA and the USSR submitted their results mainly in the field of applied column chromatography. Especially the synthesis and characteristics of stationary phases, chromatographic separation of drugs, metabolites, biologically active and endogenous compounds were the targets of the investigations. At the same time an insight into the general and theoretical aspects of chromatography, as computer assisted data evaluation, displacement chromatography, electrochemical detection, etc., is also given in the book.

A total of 54 papers has been divided into 5 chapters: "General topics", "Stationary phases for chromatography and their interactions", "Drugs, metabolites, biologically active compounds and endogenous substances", "Separation of amino acids, polypeptides and nucleotides" and "Separation of substances of various classes" yield 7, 12, 23, 8 and 4 papers, respectively.

This book gives up-to-date papers on the separation of several special and very important substance-groups, in-depth studies on the chromatography of amino acids, peptides, drugs, metabolites, nucleotides, interesting papers on stationary phases. The volume may count on the interest of chemists and biochemists working in theoretical and practical fields alike.

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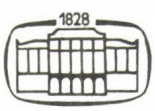

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CHROMATOGRAPHY '84

Proceedings of the Advances
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FOREWORD

In monographs, forewords have an important functional role. It is the only channel of authors to explain why the volume has turned out to be as it has. It can direct the reader in a certain trend that may hinder him in the real appreciation of the work.

In the case of proceedings, the situation is basically different. First of all, the reader is fully authorized to turn over the pages of the foreword. The authors and the papers of the present volume speak for themselves, no explanation or excuse is necessary. When one of the editors asked me to write this foreword my first reaction was to refuse the request. However, the nostalgia overwhelmed every aversion to writing because this bulky volume contains the material of the papers presented in the Advances in Liquid Chromatography which was held in Szeged, Hungary, during September 10-14, 1984, and the request recalled the best of my memory: the possibility to meet old and new friends, the numerous exciting and heated arguments during the discussions, attractive and well-working instruments, excellent books, etc. in the booths of exhibitors.

Advances in Liquid Chromatography was a very important station in the series of chromatography conferences initiated in 1981 in Szeged, Hungary. These meetings are not merely a couple of chromatographic discussions but have served to intensify the constantly growing ties between scientists unimaginable in 1981. Just one "product" or "output" of these connections is the INTERCHROM Laboratory of the Central Research Institute for Chemistry of the Hungarian Academy of Sciences and Interconcepts, Inc. (Palo Alto, Calif. USA) which

has enabled researchers working in both East and West to collaborate already producing attractive results in scale up of HPLC for biotechnology.

The strength of the connection between scientists and the conference series is also proved by the fact that the number of participants of our two latest conferences(Advances in Liquid Chromatography and Budapest Chromatography Symposium) was over 300, and there is a remarkable interest in our forthcoming meeting, New Advances in Liquid Chromatography, to be held in Szeged, 1986. It is also worth mentioning that the proceedings of the Budapest Chromatography Symposium is also very close to being published with a similar number of papers as in this present book. For technical reasons, the editors of these volumes transferred one paper of the Budapest Chromatography Symposium (written by Wojtusik et al.) to this volume, otherwise all other papers were presented in Szeged, 1984 by their authors coming from Canada, Czechoslovakia, Egypt, GDR, Hungary, Italy, Poland, Romania, Sweden, USA, USSR, and Yugoslavia.

Special thanks are due to everybody involved in the organization of the Advances in Liquid Chromatography Conference and this volume, including the participants, the sponsors and the organizers of the conference, as well as the editors, authors, and the copy editor staff of the Publishing House of the Hungarian Academy of Sciences.

Tibor Dévényi

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COMPUTER-ASSISTED ACQUISITION AND ANALYSIS OF HPLC DATA IN CORONARY AUTOREGULATION STUDY

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SUMMARY

In a study of the role of adenosine (Ado) in coronary autoregulation and vasodilation, the acquisition, storage, and High Performance Liquid Chromatographic (HPLC) data from plasma samples was facilitated with the use of both on-line and off-line computers. On-line data acquisition involved the integration of all peaks in the chromatogram of which Ado was of particular importance. The raw data, peak areas, and retention times were stored on floppy disks. An off-line computer was used for the final mathematical analysis of the data to convert the area of the Ado peak into an expression of chemical concentration; corrections for sample dilution and for addition of tritium-labeled Ado(^{3}H-Ado) were included in the calculation.

INTRODUCTION

The acquisition, storage, and analysis of large amounts of HPLC data were required for a cardiac study involving the measurement of adenosine (Ado) in porcine plasma. Data acquisition was accomplished by interfacing the ultraviolet (UV) absorbance detector of the chromatograph to a computer. The computer

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stored all raw data on floppy disks and subsequently processed the data, producing retention time and peak area information.

The plasma samples were diluted by the addition of a small volume of ^{3}H-Ado and a solution of other compounds required for sample preparation and processing. Therefore, mathematical manipulation of the Ado concentration obtained from the quantitation of the peak on the UV chromatogram was necessary to obtain values for endogenous plasma Ado concentration. An off-line computer with an appropriate program was used to mathematically correct for sample dilution and for addition of ^{3}H-Ado, and to convert the Ado concentration determined from the UV response to the amount of Ado in plasma.

INSTRUMENTATION AND METHODS

Computer Equipment

A Heathkit H11A Digital Computer, Model LSI-11 (Heathkit Company, Benton Harbor, Michigan) was interfaced to the UV detector of the chromatograph. Storage of raw chromatographic data on floppy disks was accomplished with a Heathkit H27 floppy disk drive. A video terminal (Heathkit H19) was used to display retention times, peak areas, and raw data. A memory expansion module (Heathkit H11-1) was also employed. The data acquisition system was a 16-channel multiplexer with a 12-bit A/D converter and gain amplifier (ADAC Corporation, Woburn, Massachusetts). A parallel interface module (Heathkit H11-2) was used to interface the A/D concerter to the computer data base.

Chromatographic Equipment and Methodology

The HPLC system used was a gradient liquid chromatograph with a variable wavelength UV detector from Bio-Rad Laboratories, Chemical Division (Richmond, California).

Separations of components in the plasma were performed on C-18, 5-micron, reversed-phase columns (150 x 4.6 mm) (Bio-Rad Laboratories). The columns were designed by Bio-Rad for optimal

analysis for VMA (3-methoxy-4-hydroxy mandelic acid). A guard column holder and C-8 pre-packed cartridges (4.6 mm ID x 3 cm) (Bio-Rad Laboratories) were used to protect the analytical columns.

Optimal resolution of Ado from the other plasma constituents was achieved using a step gradient elution method. The initial eluent was 98% of 0.02M KH_2PO_4 buffer (HPLC Grade, Fisher Scientific, Fair Lawn, New Jersey) in distilled, deionized water (dd-H_2O), pH 5.5, and 2% of a mixture of 60% anhydrous MeOH (HPLC Grade, Fisher Scientific) and 40% dd-H_2O (v/v). The system was run with the initial eluent for eight minutes with a subsequent step-up in 0.5 minutes to 18% of the 60% MeOH. The flow rate was 2.0 ml/min, and all separationswere performed at ambient temperature.

A micro-fractionator from Gilson Medical Electronics (Middleton, Wisconsin) was used for the fraction collection of the chromatographic effluent of samples containing 3H-Ado. The effluent was collected in 0.2 minute (0.4 ml) fractions. Liquid scintillation counting of the fractions was performed with a Mark III Liquid Scintillation System (Searle Analytic, Inc., Des Plaines, Illinois).

DATA ACQUISITION

Data acquisition involved the following: accessing the analog output of the detector (millivolts), scaling the analog voltage to a value in volts, converting the analog signal to a digital signal, and transferring the digital value to the memory in the computer (1). A parallel transmission interface with a high speed sample and hold amplifier was used to connect the output of the detector to the computer data bus. The data transfer rate of the interface module was 50 kilowords per second. The analog signal from the detector in millivolts was scaled to a voltage in units of volts with an operational amplifier. Conversion of the analog signal to a digital equivalent was accomplished with a 12-bit, 16-channel multiplexer A/D converter. The operating speed of the A/D converter was

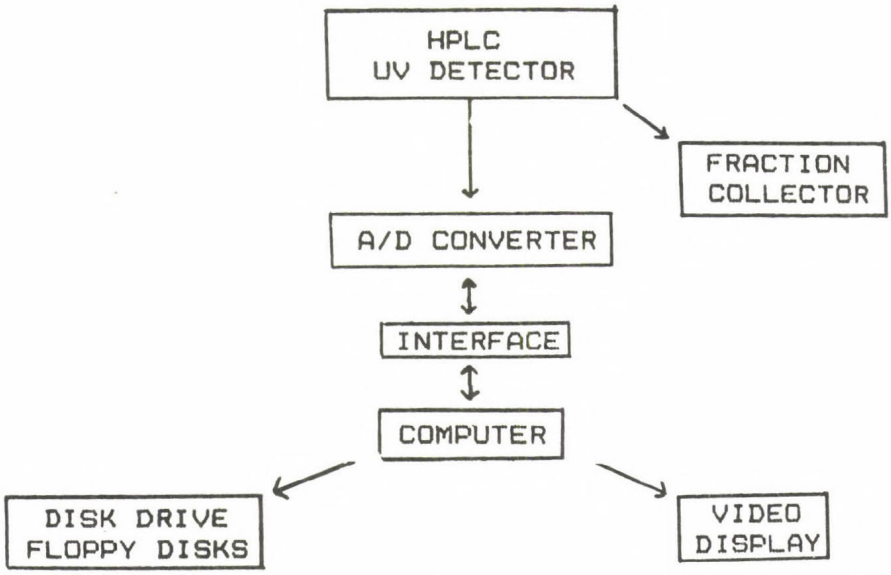

Fig. 1. Basic Schematic of the HPLC and On-line Computer

100 kHz (10 μsec, settling and conversion time). The digital values were then transferred to the computer memory expansion unit, which consisted of static memories organized in 1,024 words by four bits wide. The maximum access time of the memory expansion unit was 500 nsec. All raw data points were stored for each chromatographic run. A schematic of the basic system is shown in Figure 1. Since all raw data was stored, manipulation of peak detection could be performed at a later time. The sampling rate, which establishes the number of times the output from the detector is recorded, was 240 samples/sec. The sampling rate was a factor of 60 Hz to minimize induced noise (2)

DATA PROCESSING

Data processing involved peak detection, peak integration, tabulation of retention time and peak area data, and storage of all chromatographic data. Three parameters were used to establish the detection of a peak; the beginning threshold, the peak

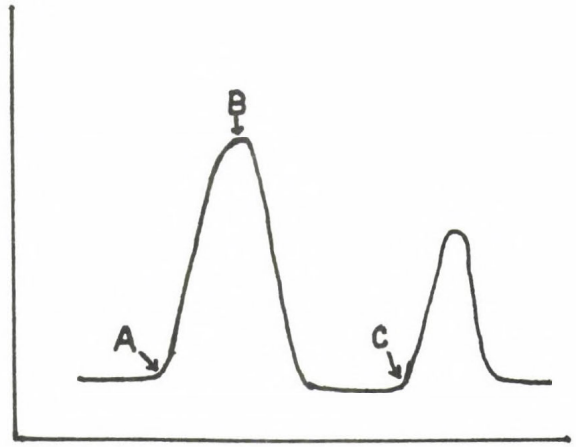

Fig. 2. Representation of a chromatogram indicating (A) Beginning Threshold, (B) Peak Threshold, and (C) Valley Threshold

threshold, and the valley threshold (Figure 2). The beginning threshold established the slope required to detect the start of the first peak. The slope was calculated between successive samples, and the starting point was detected when the slope exceeded the value calculated for the first peak. The peak threshold referred to the slope required to detect the peak time. The slope had to be less than the pre-set value for the peak threshold in order to detect a valid peak. The valley threshold was the slope required to detect the beginning of all peaks after the first peak. Therefore, a baseline shift as a result of the change in mobile phase during the step gradient did not affect the detection or the integration of the peaks eluting after the baseline shift. Also, since all raw data was stored, the peak detection parameters could be altered in post-run time and the chromatogram re-analyzed.

The data processing program also allowed the user to set the number of data points to be collected during a chromatographic run. This feature was necessary since we did not incorporate a signal that would indicate to the computer that a chromatographic run had started or was completed. Instead, the computer was started and was programmed to sound a tone at 20 seconds, and at that point to begin taking data from the detec-

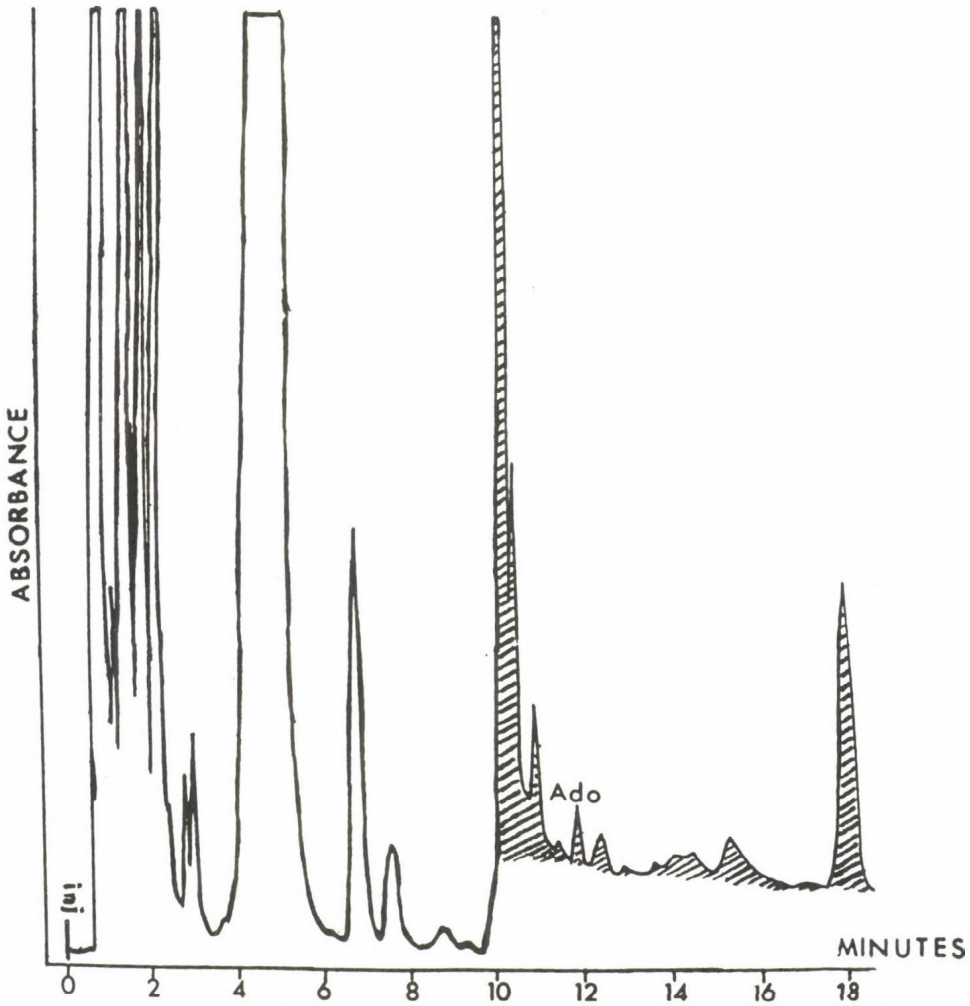

Fig. 3. Chromatogram of porcine plasma illustrating Wait Time. Retention data and peak areas were reported for peaks eluting after 10 minutes (Shaded Peaks). 10 minutes = = pre-set wait time. Chromatographic conditions: C-18 reversed-phased column (150 x 4.6 mm), C-8 guard columns (4.6 mm I.D. x 3 cm). Initial eluent: 98% of 0.02M KH_2PO_4 and 2% of 60% MeOH for 8 min, linear gradient to 18% of 60% MeOH for 10.5 min. Injection volume: 50 µl. UV absorbance monitored at 254 nm. Detector sensitivity: 0.005 a.u.f.s.

tor. At the tone, the user injected the sample into the chromatograph.

A wait time parameter was also incorporated into the data processing program. The video screen could display retention times and peak areas for approximately five minutes of the

chromatogram. Therefore the wait time specified the point in the chromatogram from which the chromatographic data was displayed (Figure 3). Data for a different section of the chromatogram was displayed by changing the wait time and re-analyzing the raw data. This parameter was useful because the peak representing Ado in the chromatogram eluted near the end of the separation, and it was therefore unnecessary to analyze data at the beginning of the run.

Peak integration was performed by using the data points established for the beginning of a peak, the highest point of the peak, and the last data point of the peak. A tangent of the backside of the peak was established and the area calculated. The data processing program also allowed the user to integrate manually peaks by telling the computer the data points that represented the beginning, the maximum height, and the end of the peak. The data points were obtained by reviewing the stored raw data. This feature was especially useful for very small chromatographic peaks which were not satisfactorily detected.

At the end of a chromatographic run, the retention times and peak areas for the peaks in the section of the chromatogram specified by the wait time were displayed on the video monitor. Since our analysis required data for only the peak representing Ado in the chromatogram, no hard copies of the data were obtained. However, the system could have been easily adapted to provide reports of chromatographic data with a suitable printer and interfacing hardware and software.

OFF-LINE DATA MANIPULATION

An off-line computer was used to do repetitious, mathematical calculations which were required to report the final concentration of Ado in plasma. The first calculation required was the determination of the exact plasma volume per blood sample drawn. The total plasma volume per sample was calculated by multiplying the weight of the drawn blood by the hematocrit of the blood. Also, the values obtained from the UV chromatograms for the concentration of Ado were not indicative of the amount

of endogenous Ado in the plasma, and since a known volume (0.960 ml) of ^{3}H-Ado and solutions of several compounds required for sample preparation were contained in the sample injected into the chromatograph, the computer calculated the appropriate dilution factor to yield exact plasma sample volume.

Tritium-labeled Ado was added to each blood sample in order to evaluate endogenous Ado loss during the sample preparation procedure. The chromatographic effluent from a separation was collected in 0.2 minute fractions, and the tritium activity (in cpm) in each fraction determined by scintillation counting. Assuming the radioactive and non-radioactive Ado was catabolized to the same extent, the percent loss of ^{3}H-Ado was used to establish the degree of endogenous Ado loss. Therefore, mathematical corrections were required to substract out the amount of ^{3}H-Ado added to a sample and to adjust the endogenous Ado concentration by the appropriate factor to reflect Ado loss during sample preparation. The cpm values of each fraction were manually entered into the computer. The computer then plotted the nuclear chromatogram and calculated the ratio of 3H-Ado cpm to the total cpm. The computer then used the cpm ratio, the plasma dilution factor, the amount of ^{3}H-Ado in the sample, and the concentration of Ado determined from the UV chromatogram to calculate the final concentration of endogenous Ado in picomoles/ml plasma.

APPLICATION

The chromatographic method, including data acquisition, processing, and analysis, as well as the method for calculating the endogenous Ado concentration was applied in a coronary autoregulation study involving domesticated swine. A typical UV chromatogram and the corresponding nuclear chromatogram of a porcine plasma sample are shown in Figures 4 and 5. The baseline shift occurring at 9.8 minutes on the UV chromatogram resulted from the step-up to 18% of 60% MeOH. The retention time of the Ado peak on the UV chromatogram was 12.0 minutes. The

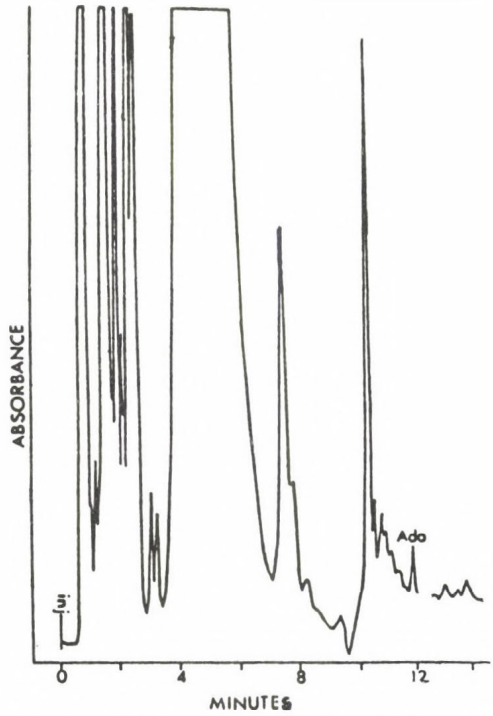

Fig. 4. UV Chromatogram of a porcine plasma sample. Chromatographic conditions same as Fig. 2, except final eluent (18% of 60% MeOH) maintained for 5.5 min

slightly longer time of the Ado fractions on the nuclear chromatogram reflected the additional time and volume of effluent between the UV detector and the fraction collector. Since Ado was the only tritium-labeled compound used, the nuclear chromatogram contained only one major peak; i.e., ^{3}H-Ado. The small peak eluting at one minute (Figure 5) represented the solvent front and resulted from hydrolysis of the tritium label. The amount of Ado representative of the Ado peak in the UV chromatogram was determined from the peak area obtained with the on-line computer. The nuclear chromatogram was plotted with the off-line computer by the method mentioned previously. The ratio of ^{3}H-Ado cpm to the total cpm in the fractions of the nuclear chromatogram (Table 1) for the plasma sample in Figures 4 and 5 was 0.93, indicating that a 7% loss of Ado occurred during sample preparation. The cpm ratios, along with the input data (Table 2A), were used by the computer to calculate the corresponding output data and the concentration of endogenous plasma

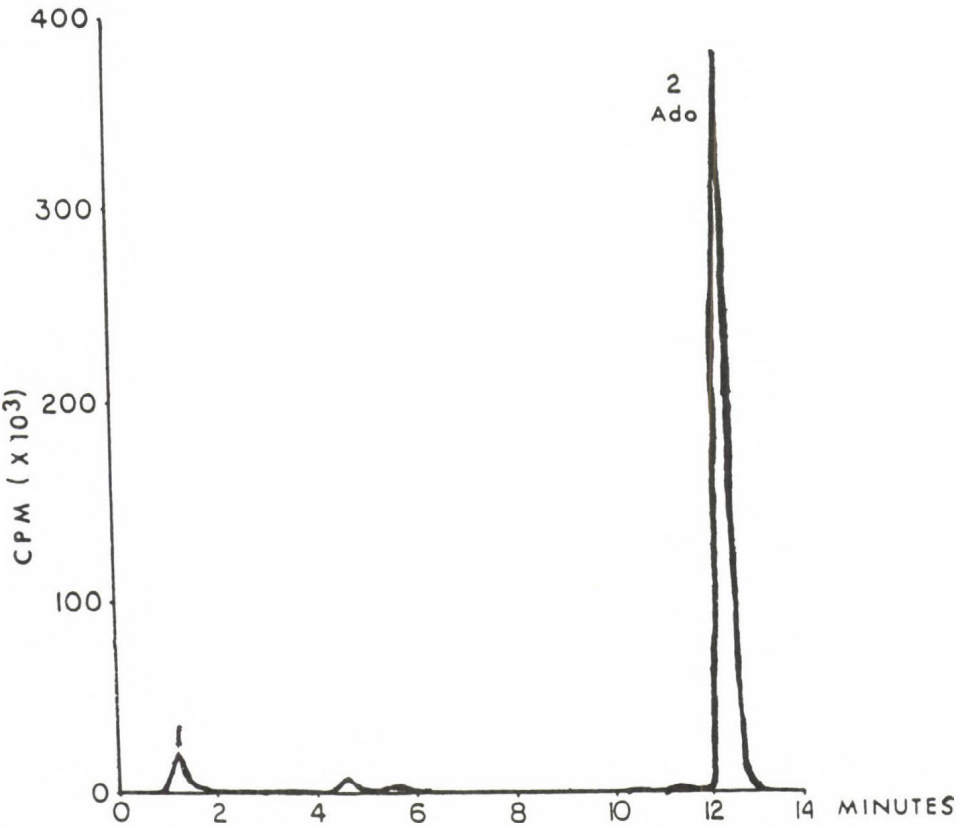

Fig. 5. Nuclear Chromatogram of a porcine plasma sample. Chromatographic conditions for the samples are described in the legend of Fig. 2. Chromatographic effluent fractionation: 75 consecutive 0.2 min (0.4 ml) fractions. Liquid scintillation counting time: 1 min

Table 1. CPM ratio

Total CPM=642142	
Total ADO CPM = 596921	
ADO CPM/Total CPM = 0.93	

CPM = counts per minute
ADO = adenosine

Ado (Table 2B). The information in Tables 1 and 2 is in a format representative of the computer printouts obtained for

Table 2. Input and output data for the determination of endogenous plasma ADO concentration

A. Input data

a. HCT = 34.0
b. Blood WT = 3.1615
c. ADO STD CPM = 45000000
d. ADO STD PMS = 1553.0
e. [ADO]-UV = 53.1

B. Output data

f. Predicted ADO CPM/50 µl Sample = 738531
g. PMOL Hot ADO/50 µl Sample = 20.6
h. Corrected PMoles ADO/50 µl Sample = 40.2
i. [ADO] PM/ML = 1174.2

a. HTC = hematocrit
b. Blood Weight in grams
c. ADO STD CPM = cpm in 50 µl of pure, stock 3H-ADO
d. ADO STD PMS = pmoles Ado in 50 µl of pure, stock 3H-ADO
e. [ADO]-UV = pmoles Ado/50 µl injection volume from UV chromatogram
f. Predicted ADO CPM/50 µl Sample = Ado cpm corrected for sample dilution
g. PMole Hot ADO/50 µl Sample = pmoles ^{3}H-Ado in 50 µl of the injected sample
h. corrected PMoles Ado/50 µl Sample = pmoles Ado/50 µl
i. [ADO] PM/ML = Endogenous Ado concentration in plasma

Table 3. Calculations of endogenous ADO concentration

I. Calculation of the plasma dilution factor (Dil.F.)

A. Blood wt x (1-%HCT) = ML, Plasma Extract
Plasma Extract = Whole Plasma + Endogenous Compounds (0.960 ml)

B. (ML, Plasma Extract + 0.96)/Ml Plasma Extract = Dil.F.

II. Calculation of Endogenous Ado Concentration in Plasma

A. $\frac{\text{Predicted ADO CPM}}{\text{50 µl Sample}}$ = (Total ADO CPM/Dil.F.) x 0.05

B. $\frac{\text{PMol Hot ADO}}{\text{50 µl Sample}} = \frac{\text{ADO STD PMS}}{\text{ADO STD CPM}}$ x Total ADO CPM

C. $\frac{\text{Corrected PMoles ADO}}{\text{50 µl Sample}}$ = [ADO]-UV - $\frac{\text{PMol Hot ADO}}{\text{50 µl Sample}}$

D. $\frac{\text{PMoles ADO}}{\text{PL Plasma}} = \frac{\text{Corrected PMoles ADO}}{\text{50 µl Sample}} \times \frac{\text{20 µl}}{\text{ML}}$ x Dil.F.

an analysis. The equations used to calculate the Ado concentration are shown in Table 3.

CONCLUSION

An on-line and off-line computer facilitated the acquisition, storage, and analysis of large amounts of chromatographic data. A computer that was interfaced on-line with the UV detector of the chromatograph was used to acquire and process the raw data from the UV detector to yield retention times and peak areas for the peaks of interest in the chromatograms. All raw and chromatographic data were stored on floppy disks for each plasma sample. The advantage of storing raw data was to allow the user to alter peak detection parameters in post-run time.

The off-line computer was used to produce the nuclear chromatograms and to facilitate the repetitious, mathematical calculations which were required to obtain the concentration of endogenous Ado in the plasma.

The methods of data acquisition, storage, and analysis were applied to a cardiac study involving the measurement of Ado in a large number of samples of porcine plasma. The on-line computer system, even though used exclusively for the cardiac study, could be employed for any HPLC analysis. The off-line computer, used for the mathematical data manipulation, reduced greatly the time required for a complete analysis.

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DESCRIPTION OF CHROMATOGRAPHIC ANALYSIS BY THE MEASURE THEORETICAL MODEL

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SUMMARY

For the purpose of modelling chromatographic analysis by the measure theory we must first give the mathematical definition of molecules, of their chemical and chromatographic properties, of the chemical and chromatographic components and the component quantities. Using these basic concepts a chromatogram can be described as a mixture distribution of the normalized chromatographic distributions of the chemical components in the sample weighted by the chemical composition of the sample. An extension of the one-dimensional model to a two-dimensional model for thin-layer chromatography will be given.

INTRODUCTION

Chromatographic analysis

The process of chromatographic analysis is very complex from the point of view of both theory and practice; therefore, an exhaustive discussion is impossible. In our introductory treatment only some features characterizing the chromatographic analysis from the point of view of system theory and relevant for the formulation of the mathematical model will be discussed.

In chromatographic analysis the chromatographic system transforms the sample into a chromatogram, which then is interpreted as the chemical composition of the sample. For interpretation one must know the chromatogram as an output belonging to

the sample of known chemical composition, which is an input under fixed chromatographic performance parameters. This knowledge enables us to infer the chemical composition of an unknown sample from the chromatogram measured in analysis.

The input of the chromatographic analysis is the chemical composition of the sample which is known in calibration, and unknown is analysis. The output of the process is the chromatographic composition of the sample. The model of the chromatographic analysis describes the relationship between the chromatogram as an output and the chemical composition as an input.

Basic ideas for modelling the chromatographic analysis

The number of publications dealing with the different problems in the mathematical modelling of the chromatographic analysis processes is rather vast; see e.g. /1/-/4/.

In spite of the great number of books and papers appearing on the subject, practically no attention has been paid to the input-output models of chromatographic systems, from the point of view of quantitative analysis.

The chemical distribution of the material and the material quantity is based on the chemical identity of molecules. Thus, the chemical distribution of the material means that the material is distributed among its chemical components, and the chemical distribution of the material quantity means that the quantity of the material is distributed among the component quantities.

In chromatographic analysis, besides the chemical distribution, there is another distribution, the chromatographic distribution of the sample. The chromatogram is the observed chromatographic distribution of the sample. The chromatographic distribution of material and material quantity is based on the chromatographic behaviour of the molecules. The chromatographic distribution of the material is the material distribution on the chromatographic column. The chromatographic distribution of material quantity is the distribution of the material quantity shown by the detector in the chromatographic distribution of material.

On the basis of the chemical and chromatographic distributions of the material and material quantity the chromatographic-chemical distribution can be defined which is equivalent to the chromatographic distribution of pure chemical components.

A single chemical component will produce a chromatographic peak which can be regarded as the chromatographic distribution of a given component. The chromatogram in general can be interpreted as the superposition of the chromatographic peaks of the chemical components found to be present in the sample. Thus the chromatogram is actually the chromatographic distribution of the sample quantity which can be described as a mixture of the chromatographic distributions of the chemical components weighted by the chemical distribution of the sample quantity.

The mathematical description of the above statement will be given using a measure theoretical model. This interpretation requires the concepts of sample distribution and sample quantity distribution. For both types of distributions the chemical and the chromatographic distributions can be defined.

The measure theoretical modelling of chromatographic analysis means establishing a relationship between the chemical and the chromatographic distribution of the material and material quantity. The relation can be expressed by the following function:

Chromatographic distribution of the sample (i.e. the chromatogram) = f (chemical distribution of the sample)

This expression gives the general input-output model of the chromatographic analysis system, where the chemical distribution of the sample encompasses the input and the chromatogram the output. In a majority of cases, however, this connection can be given in the following form:

$$\left\{\begin{matrix}\text{chromatographic}\\ \text{distribution of}\\ \text{the sample}\end{matrix}\right\} = \left\{\begin{matrix}\text{chromatographic}\\ \text{distribution of}\\ \text{the components}\end{matrix}\right\} \begin{matrix}\text{weighted}\\ \text{by}\end{matrix} \left\{\begin{matrix}\text{chemical}\\ \text{distribution}\\ \text{of the sample}\end{matrix}\right\}$$

in other words

$$\text{chromatogram} = \left\{\text{component peaks}\right\} \quad \begin{matrix}\text{weighted} \\ \text{by}\end{matrix} \quad \left\{\begin{matrix}\text{chemical} \\ \text{composition} \\ \text{of the sample}\end{matrix}\right\}$$

In the further discussion the mathematical formulation of these principles will be given.

CHROMATOGRAPHIC ANALYSIS

Basic concepts in the measure theoretical model

The measure theoretical model describing chemical and chromatographic distributions of the material and material quantity requires the introduction of the following basic concepts.

The smallest element of the sample to be investigated effecting the chemical and chromatographic behaviour is the molecule (or ion). In the measure theoretical model it is called molecule and marked m.

The material is investigated from chemical and chromatographic points of view, thus the chemical and chromatographic properties of the molecule are modelled. The concept property of molecule is defined in the model. The chemical property of the molecule is marked by $\varphi^{c}(m)$, while the chromatographic property by $z^{a}(m)$; i.e., the properties give the values of the chemical and chromatographic properties of the selected molecule.

The totality of molecules makes up the investigated material or sample. Material is interpreted as a set of molecules, i.e., for material M, $m \in M$. A subset of the set of molecules, i.e., a subset of material is called component and designated by C, $C \subseteq M$.

In order to give the measure theoretical definition of material quantity a non-negative measure is interpreted for component C which will be called component quantity and marked q(C).

Chemical distribution of the sample

The basic concepts introduced above are used to define the chemical distribution of the material and material quantity.

Let the chemical property of the molecule be equivalent to the next statement:

$$\varphi^{C}(m) = \phi_n^C$$

which means that the chemical property $\varphi^{C}(m)$ of molecule m has the value of ϕ_n^C. For example, if the chemical property $\varphi^{C}(m)$ means the chemical identity of the molecules then ϕ_n^C means that the molecule is benzene, toluene, etc.

The chemical components are defined as sets of molecules according to their chemical property:

$$C_n^C = \{m : \varphi^{C}(m) = \phi_n^C\} \qquad \text{for } n=1,\ldots,N,$$

In other words, the n-th chemical component C_n^C is the set of all molecules m that display ϕ_n^C as a value to the chemical property $\varphi^{C}(m)$. This means that, for example, benzene, as a component consists of molecules with the chemical identity of benzene:

$$C^C_{\text{benzene}} = \{m : \varphi^{C}(m) = \text{benzene}\}$$

The discrete character of the chemical property defined above implies discrete chemical components.

The chemical distribution of the material can be interpreted as the totality of chemical components in the investigated material, i.e., $C^C_1,\ldots,C^C_N$.

If the property defining the chemical components is introduced

$$\rho_n^C(m) = \begin{cases} 1 & \text{if } m \in C_n^C \\ 0 & \text{otherwise} \end{cases}$$

then the qualitative chemical composition of the material can be given by a chemical qualitative composition vector:

$$\underline{\rho}^C = \begin{pmatrix} {}^C\rho_1 \\ \vdots \\ {}^C\rho_N \end{pmatrix} .$$

The quantity of components in the material is defined as the chemical measure on the components $q^C(C^C{}_n)$; i.e., $q^C(C^C{}_n)$ denotes the chemical quantity of the chemical component. This chemical quantity can be expressed by mass, volume, etc.

On the basis of the chemical quantity of the chemical components the chemical distribution of the material quantity can be defined. The chemical distribution of the material quantity corresponds to the chemical quantities of components $q^C(C^C{}_1)$, $q^C(C^C{}_2),\ldots,q^C(C^C{}_N)$ for which the following expression holds:

$$\sum_{n=1}^{N} q^C(C^C{}_n) = q^C(M) .$$

The quantitative chemical composition of the material can be given by the chemical quantities of the chemical components in the material in the form of a quantitative chemical composition vector:

$$\underline{q}^C = \begin{pmatrix} q^C(C^C{}_1) \\ \\ q^C(C^C{}_N) \end{pmatrix}$$

Chromatographic distribution of the sample

In the measure theoretical model the chromatographic distribution of the material can be described as follows.

First a chromatographic property of the molecule $z^a(m)$ must be defined. This expresses the retention characteristics of the molecules. The statement

$$z^a(m) < r$$

expresses that retention characteristic $z^a(m)$ of molecule m is less than r. It is to be noted that the statements of the form

$$z^a(m) = r$$

cannot be applied from a mathematical point of view for continuous retention properties $z^a(m)$.

The retention property is considered one-dimensional for column chromatographic and TLC processes and two-dimensional for thin-layer chromatography. First the one-dimensional case will be discussed and then the description extended for two-dimensional case.

Using the chromatographic property the chromatographic components can be defined as sets of molecules having a retention characteristic less than r:

$$C^a_r = \{m : z^a(m) < r\}, \qquad r \in R.$$

The above defined chromatographic characteristic can be an arbitrary property, either discrete or continuous. Correspondingly, the chromatographic components of the sample can also be continuous.

It is a typical feature of all chromatographic analysis processes that the resulting discrete distribution of the components is modified by unavoidable dispersion. Due to this the chromatographic distribution should be regarded as continuous. The proposed measure theoretical model is also suitable to describe discrete distributions. The discussion of this, however, would far exceed the coverage of this paper.

The chromatographic distribution of the sample is the totality of the chromatographic components as a function of the retention property.

For the chromatographic components of the material a chromatographic measure $q^a(\cdot)$ can be defined: $q^a(C^a_r)$ expresses the chromatographic quantity of chromatographic component C^a_r. The chromatographic measure is the value of the detected signal, for example, the absorbance, amperage, etc., or the peak area.

Once the chromatographic quantities of the chromatographic components are defined, the chromatographic distribution of the chromatographic quantity of material can also be formulated. For continuous retention characteristic the chromatographic distribution of the material quantity is given by:

$$G^a(r) = q^a(C^a_r) \quad , \quad r \in R$$

or by $g^a(r)$ the chromatographic density function, the derivative of the chromatographic distribution function. It can easily be seen that the chromatographic density function of material quantity is the mathematical description of the chromatogram.

Chromatographic distribution of components

Besides the chemical and the chromatographic distributions of the material and material quantity the chromatographic-chemical distribution of the chemical components can also be defined.

The chromatographic-chemical component is defined on the basis of joint chemical and chromatographic properties of the molecules as the following set of molecules:

$$C^{ac}_{rn} = \{m : \varphi^c(m) = \phi^c_n \quad z^a(m) < r\}$$

$$n = 1,\ldots,N \; ; \quad r \in R,$$

i.e., a set of molecules identified as the n-th chemical component and with a retention characteristic less then r.

The components thus defined can be used to determine the chromatographic distribution of the chemical components, as a totality of all sets C^{ac}_{rn} for a given n and for every r. The

chromatographic distribution of the chemical components means the distribution of the pure chemical component in the chromatographic system.

The chromatographic-chemical component can also be characterized by the quantity $q^a(C^{ac}_{rn})$. In the case of continuous retention properties the chromatographic distribution of the chromatographic quantity of the chemical component is given by:

$$G^a_n(r) = q^a(C^{ac}_{rn}) \quad r \in R; \quad n=1,\ldots,N.$$

The chromatographic distribution function of the chemical component of its derivate is the density function $g^a(r)$. The chromatographic density function of a chemical component is the mathematical description of the chromatogram belonging to a pure chemical component.

Model of the chromatographic process

As stated above the chromatogram is modelled by the chromatographic density function of material quantity, $g^a(r)$. If we assume that no interaction exists between the chemical components, i.e.,

$$C^a_r = \bigcup_{n=1}^{N} C^{ac}_{rn}$$

then

$$q^a(C^a_r) = \sum_{n=1}^{N} q^a(C^{ac}_{rn})$$

which implies that

$$g^a(r) = \sum_{n=1}^{N} g^a_n(r)$$

In other words, the chromatogram is a sum of the chromatograms of pure components.

Let $g_n^{ac}(r)$ be the normalized chromatogram of the n-th chemical component determined according to the following formula:

$$g_n^{ac}(r) = \frac{g^a{}_n(r)}{q^c(C^c{}_n)}$$

Based on the above equations for the generalized description of a chromatogram we obtain the following relationship:

$$g^a(r) = \sum_{n=1}^{N} g_n^{ac}(r) \cdot q^c(C^c{}_n),$$

which states that a chromatogram is a mixture of the normalized chromatograms of pure components weighted by the quantity of these components.

Effect of the chromatographic process parameters

In practice the parameters effecting the chromatographic process must be taken into consideration. Some important process parameters characterize separation, such as the column packing, mobile phase, temperature, pressure; some characterize detection, e.g., the type of detector, sensitivity, etc. The measure theoretical description introduced herein implicitly embodies these chromatographic process parameters.

The effect of the parameters characterizing chromatographic separation appears in the chromatographic property z^a, i.e.,

$$z^a = f \text{ (chromatographic separation parameters)}$$

where $f(\cdot)$ marks a general retentionship, the specific form of which must be determined for each particular case.

The effect of different detection parameters appears in the chromatographic quantity, i.e.,

$$q^a = f \quad \text{(detection parameters)}$$

where $f(\cdot)$ again is a general function.

It is obvious that the dependence of the retention characteristic and the chromatographic quantity from the process parameters can be determined and built into the measure theoretical model. This model expresses the results of a chromatographic process under constant process parameters.

Calibration and analysis

The measure theoretical description of the chromatograms can be regarded as the input-output model of a chromatographic analysis.

In order to apply the model to practice, it is necessary to identify the model by calibration. In calibration the normalized chromatograms of pure components are determined from samples of known chemical composition and from their measured chromatograms, i.e., from the chromatographic distribution of sample quantity.

In analysis the chemical composition of the sample is determined from the chromatogram and from the knowledge obtained in calibration from the normalized chromatograms of pure components.

Two dimensional thin-layer chromatography

The "one-dimensional" or "column" chromatographic model introduced above can easily be extended for the two-dimensional case of thin-layer chromatography.

In the description of thin-layer chromatographic processes the only difference with respect to the one-dimensional model is that the chromatographic property of the molecule, i.e., the retention characteristic is now two-dimensional. The retention characteristic can be expressed by a two-dimensional vector, i.e.:

$$\underline{z}^a(m) = \begin{pmatrix} z^a{}_x(m) \\ z^a{}_y(m) \end{pmatrix},$$

where $z^a{}_x(m)$ and $z^a{}_y(m)$ are the components of the retention characteristic falling in x and y directions.

In this case the chromatographic components can be defined as the following sets:

$$G^a{}_{x,y} = \{m : z^a{}_x(m) < x, \quad z^a{}_y(m) < y\} \qquad \binom{x}{y} \in R^2.$$

The two-dimensional chromatographic distribution function of the chromatographic quantity of material is:

$$G^a(x,y) = q^a(C^a{}_{x,y}), \quad \binom{x}{y} \in R^2.$$

The derivate of this gives the chromatographic density function of the material quantity, the mathematical description of thin-layer chromatograms. Similarly to the one-dimensional case the chromatogram is the sum of the normalized chromatograms of pure components weighted by the quantities of the chemical components, that is

$$g^a(x,y) = \sum_{n=1}^{N} qn^{ac}(x,y) \cdot q^c(C^c{}_n).$$

CONCLUSIONS

The measure theoretical description of chromatographic analysis processes introduced herein has two merits. First this input-output model of the complex chromatographic process only reflects the relevent results of the process; the model contains laws implicitly governing the chromatographic processes. Its simplicity, however, allows us to conclude that the follow-

ing basic concepts characterize all chromatographic processes: molecules, chemical and chromatographic properties of the molecules, chemical and chromatographic components, and the chemical and chromatographic quantities of the components. Chromatographic process parameters are included in the chromatographic property, while detection parameters are in the chromatographic quantity.

Another advantage of the measure theoretical description of a chromatographic analysis is found in its generalized form. A generalization with respect to all analytical chemical investigations can easily be made since all analytical chemical methods can be described using a similar approach (see /6/). The results of the present study are considered as further steps toward the unified discussion and comparison of different analytical chemical methods which is our aim. These questions will be the scope of our future studies.

SYMBOLS

C	component
C^a	chromatographic component
C^c	chemical component
$f(\cdot)$	function in general
$g^a(r)$	chromatographic density function
$g^{ac}(r)$	normalized chromatographic density function
$g^a(x,y)$	thin-layer chromatographic density function
$G^a(r)$	chromatographic distribution function
$G^a(x,y)$	thin-layer chromatographic distribution function
m	molecule
M	sample, material
n	index of the chemical component
N	number of chemical components
q(C)	quantity of the component

$q^a(C)$	chromatographic quantity of the component
$q^c(C)$	chemical quantity of the component
$z^a(m)$	chromatographic (retention) property of the molecule
r	value of the chromatographic property
R	one-dimensional Euclidean space
R^2	two-dimensional Euclidean space
x	value of the chromatographic property in direction x
y	value of the chromatographic property in direction y
$\varphi^c(m)$	chemical property of the molecule
ϕ_n^c	value of the chemical property
$\rho_n^c(m)$	property defining the chemical component

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DISCOVERY OF A NEW MOLECULAR PROBE FOR OPTIMIZATION OF DETECTABILITY AND SELECTIVITY IN HPLC

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High-performance liquid chromatography (HPLC) has revolutionized the science of chemical separations. The compounds that once were considered too difficult to separate because of polarity, volatility, and other considerations can now be separated easily within a reasonable period of time. This advancement is hindered by the nonuniformity of columns (1). The HPLC columns with the same stationary phase exhibit significant variabilities from one manufacturer to another and even from the same manufacturer. Assuming all other conditions are constant, variabilities can occur even in the packing process. The commonly used molecular probes are insufficient to evaluate these variabilities. The search for a more selective molecular probe led to a compound that can help evaluate variabilities in C-8 columns. This probe was also found useful in defining optimum detectability and selectivity of the separation.

EXPERIMENTAL

These evaluations were carried out using a Waters liquid chromatograph and columns from various manufacturers. The columns used for in-depth investigations were 25 cm long with i.d. of 4.6 mm (Whatman Chemical Separations Inc., Clifton, N.J.). The detector was set at 254 nm and the system was operated at 25°C with the appropriate mobile phase.

THEORY

In chromatography it is important to obtain the minimum peak width and the maximum peak height for a given component. We know the peak width can be related to the number of theoretical plates of a column, N, by the following equation (2):

$$N = 16\ (V_R/W)^2 \qquad (1)$$

Where V_R = Retention volume and W = Peak width at the base

$$\text{or}\quad W = 4V_R/N^{1/2} \qquad (2)$$

Since $V_R = V_M(1+k')$, where V_M = column dead-volume; the peak width, W, can be related to k', the capacity factor, as follows:

$$W = (4/N^{\frac{1}{2}})V_M(1+k') \quad (3)$$

Peak height, A, on the other hand, can be related to the coefficient of variation (CV) of the analytical determination by the following equation (3):

$$CV = (100/\sqrt{8})\ (\chi/A) \quad (4)$$

Where χ = baseline noise and A/χ = signal to noise ratio

From the simple theoretical discussion given above, it is clear that obtaining minimum peak width requires low k' or V_R, large N, and low V_M. To enhance detection it is necessary to lower baseline noise and improve the value of the coefficient of variation. Hence, to optimize detectability and selectivity, it is important to have a thorough understanding of the HPLC equipment and the stationary phase dynamics of the column used.

RESULTS & DISCUSSION

In the early sixties, it was pointed out by Ahuja *et al* (4,5) that the stationary phase in gas-liquid chromatography is a dynamic system which is prone to change with the composition of the mobile phase. This is specially critical in high pressure liquid chromatography. The column changes because various components arising from solvents and samples are retained by the column. Also, it is not uncommon to lose resolution with a given column over a period of time. This can be caused by a loss of small amounts of stationary phase with simultaneous exposure of silanol sites. One such situation was investigated with a C-18 column, which on aging showed loss of resolution for two closely resolved components. This column was found to contain more than the manufacturer's specification of theoretical plates. Addition of a small amount of diethyl amine in lieu of inorganic buffer yielded a separation that was better than before, thus providing the basis for use of competing amines (6).

A select review of a technical report (7) listing the retention behavior of various C-8 and C-18 columns revealed the following interesting information:

a. Two C-18 columns viz. μBondapak and Partisil C-18 columns are different by an order of magnitude in k' value for anthracene from Nucleosil, Ultrasphere, and Zorbax.

b. C-8 columns are also not similar e.g., only Zorbax C-8 column shows higher k' for diethyl phthalate than the C-18 column. A similar reversal is observed with Nucleosil C-8 columns for toluic acid. For both of these separations, Zorbax C-8 column exhibits ~ 3 times the k' value of Nucleosil column.

The above review points to significant variabilities in columns. The following considerations are important for improving our understanding of column performance and selectivity (8):

Property of silica gel:	particle shape
	particle size ($\bar{x}$ and distribution)
	pore size
	pore volume
	surface area
Column characteristics:	stationary phase (C-18, C-8, C-3) -
	mono-, bi- or tri-functional;
	straight chain or branched
	surface coverage [%C, moles Si/m^2]
	end capped or not
	void volume
	flow resistance
	pressure requirement
Chromatograms:	efficiency - plate number N per column*
	peak asymmetry
	k' acids, bases, neutrals, etc.
	α acids, bases, neutrals, etc.

* For select solutes

The following conclusions can be made about properties of silicas based on various studies (9-12):

1. Chromatographically determined particle size of various silicas varies from 7.6 μm to 12.2 μm.

2. The surface area of silicas varies from 300 to 420 m^2/g with pore diameter range from 56 to 102 °A.

3. The packing factor for 5 μm silica varies from 0.76 to 1.67.

4. The pH of aqueous suspension of silicas varies from 3.8 to 9.9.

5. The pore volume varies from 0.49 to 1.48 cm^2/g and only 19 to 33% of the pore volume is occupied.

6. Methyl red absorption tests show Nucleosil has 26 times greater absorption capacity than Zorbax.

Table 1

Typical Manufacturer's Data[a] for C-8 Column

Particle size:	10.0 μm
Void volume:	2.68 ml
Length:	250 mm
Diameter:	O.D. 6.35 mm; I.D. 4.60 mm
Sample volume :	10.0 μl
Flow cell volume:	8.0 μl
Flow rate:	1.00 ml/min
Detector wavelength:	254 nm
Pressure:	700 psig
Mobile phase:	80:20 (v/v) Methanol - Water

	Benzene	Naphthalene	Biphenyl
Retention time (t_R, min)	4.53	5.90	7.31
Peak width (W60%,min.)	0.102	0.139	0.173
Efficiency[b]	7890	7207	7142
Capacity factor(k'):	0.69	1.20	1.73
Asymmetry ratio:	1.41	1.33	1.29
Resolution ratio:	5.52	4.45	
Av. plates/meter:	29,800		

[a]Whatman Chemical Separations Inc., Clifton, N.J.

[b]Efficiency (theoretical plate number), $N = 4 \left(\frac{t_R}{W60\%} \right)^2$

It is known that surface silanols can interact strongly with electron rich atoms in groups such as $-C\equiv N$, $-NO_2$, $-CH_2OH$ (13). In addition, organic solvents can compete with solutes for silanol sites and, as noted earlier, stationary phase composition can be influenced by the composition of the solvent system and column equilibration times. End-capping or silylating free silanol groups can minimize interactions of some compounds (14). These observations point to the need for a thorough investigation and understanding of columns prior to their use.

Various columns were evaluated in light of the above comments for a separation that entailed a mixture of acidic, basic and neutral compounds. The columns that were not end-capped were found unsuitable for further investigations. From the remaining, one manufacturer's column was selected for in-depth investigation. The type of data provided by this manufacturer is given in Table 1. It is readily apparent that these probes are similar in structure, i.e., they are all aromatic hydrocarbons and the effect of polar groups cannot be determined with this approach.

Nitrobenzene has been used for the evaluation of residual silanol groups. Therefore, it was selected to evaluate three columns from different batches with a mobile phase of n-heptane to determine the differences in terms of residual silanol sites in these columns. The results are given in Table 2. The data do not permit clear differentiation, e.g. column 1585 has the highest number of theoretical plates, a desirable characteristic for a column, but it gives the same peak width for nitrobenzene as column 1887.

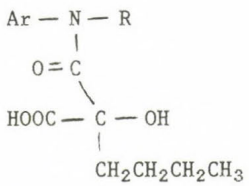
Table 2

Evaluation of C-8 Columns* with Nitrobenzene

	Column Number		
	IR 1585	IR 1646	IR 1887
Retention time (t_R, minutes)	5.39	5.71	4.47
Peak width (W,minutes)	0.47	0.54	0.47
Retention (t_B) benzene	3.35	3.31	3.13
Capacity factor (k')	0.6	0.7	0.4
Theoretical plates (N)	2027	1789	1447

*Columns 10 μm, C-8, 25 cm; mobile phase n-heptane, 1 ml/minute; amount injected: 0.04 μg (15 μl)

The same three columns could be differentiated more clearly with the new molecular probe (SA-1). It has the following structure and characteristics:



a. Aromatic character

b. Carbonyl group and substituted nitrogen in its structure

c. Hydroxyl and carboxyl groups on the same asymmetric C atom

d. A short carbon chain (C_4)

The data with the new molecular probe is given in Table 3. With a mobile phase containing 41:59(v/v) acetonitrile-0.02M acetate buffer (pH 4.1), significant differences can be observed between the three columns. At 0.1 µg level, the new molecular probe was not detected with column 1887, whereas the other two columns gave a peak for it. With column 1646 the peak tails and has a width two times that of column 1585. It should be noted that the k' is slightly lower with column 1585 than with column 1646; however, the number of theoretical plates is significantly higher (~3.5x) with this column. (The number of theoretical plates was calculated for tailing peak with column 1646 to provide a relative value).

Table 3

Evaluation of C-8 Columns* with New Probe (SA-1)

	1585	1646	1887
Retention time (minutes)	1.36	1.50	Not Detected[b]
Peak width (minutes)	0.20	0.41[a]	Not Detected[b]
Capacity factor (k')	1.9	2.2	---
Theoretical plates (N)	740	214	---

*C-8(10 µm/25 cm); 0.1 µg Molecular Probe injected; mobile phase-41:59(v/v) acetonitrile-0.02M acetate buffer (pH 4.1), Flow rate - 2.4 ml/min

[a] tails

[b] 1µg sample gives t_R = 1.73 min; W = 2.36 (large tail); k' = 2.7

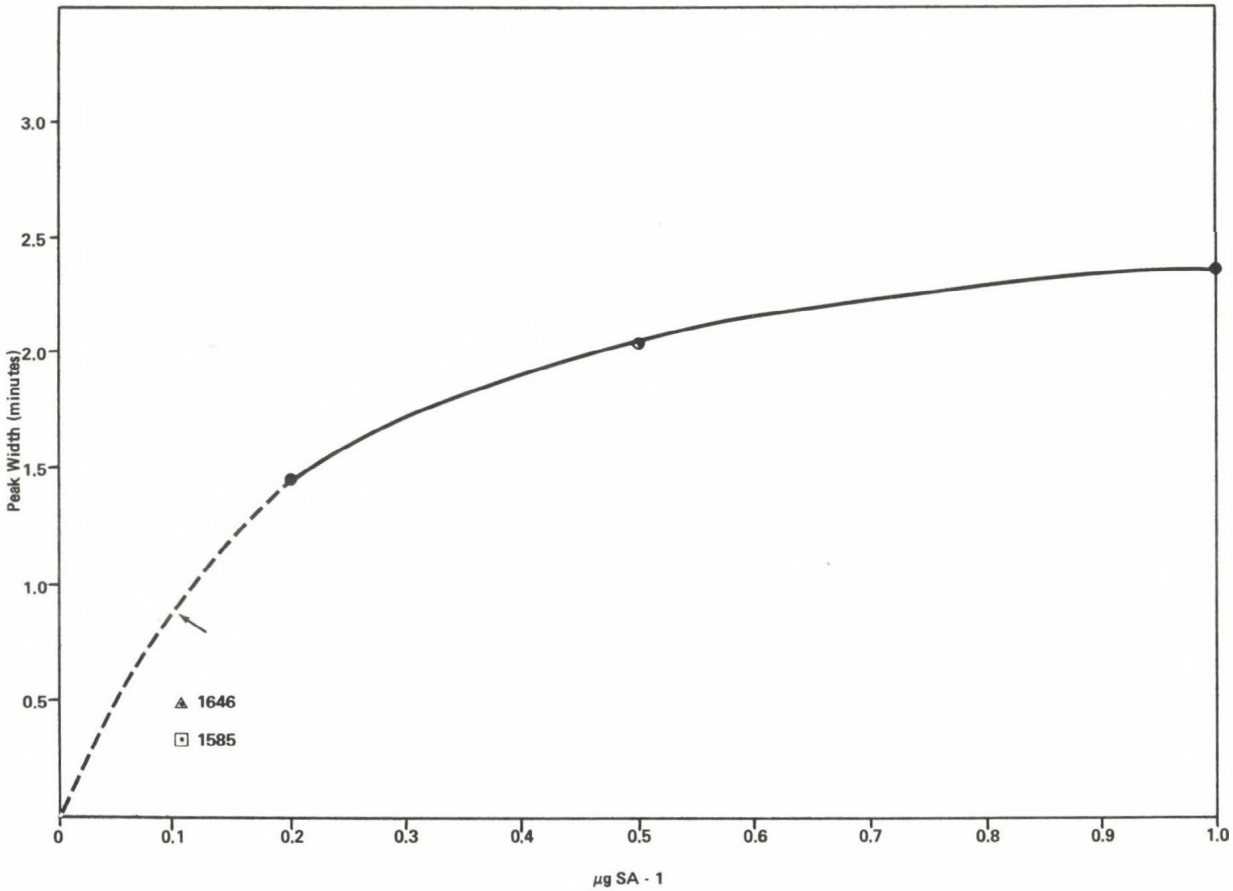


Figure 1 Relationship of Peak Width and Injected Amount of SA-1 with Column 1887

As can be seen from the data in Table 3, column 1887 behaves very differently from the other two in that 0.1 μg of the new probe is not detected. It should be noted that the amounts of probe from 0.2 μg and upwards were detected. A plot of this data is given in Figure 1. When the curve is extrapolated down to 0.1 μg level, a peak width of 0.90 minutes is indicated for this column. The peak widths obtained for the other columns are also shown in the figure for reference. These data show that detectability of a component at low levels (0.1 μg or less) can vary from one batch of columns to the next. Detectability decreases because there is a concomitant loss of peak height due to peak broadening. As the peak broadens, the resolution for closely resolved peaks is affected negatively; this can result in complete loss of resolution.

Three experimental columns were prepared in cooperation with Whatman Inc. to evaluate the effect of end-capping or minimization of residual silanol groups on detectability of the molecular probe. With

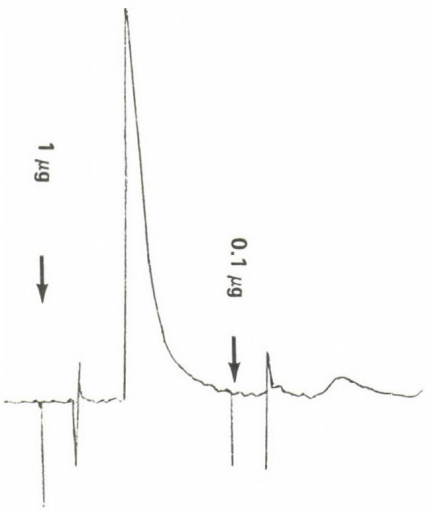


Figure 2

Chromatogram of SA-1 with Experimental Column #1
(No End-Capping)

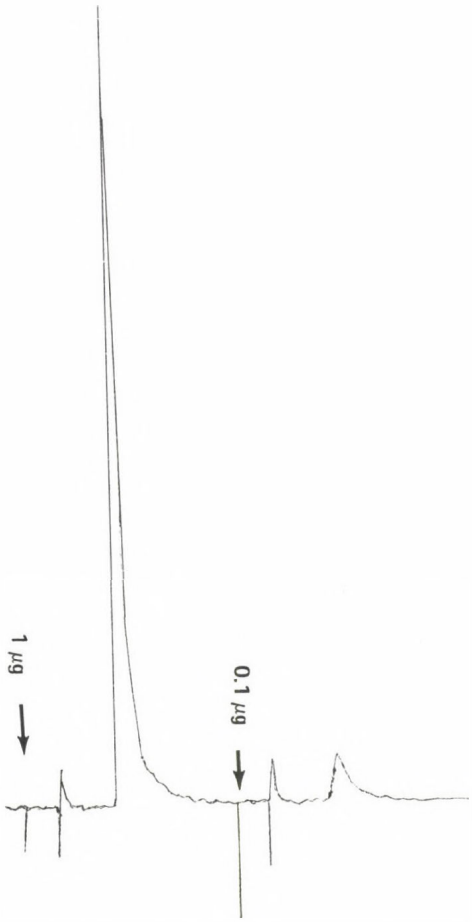


Figure 3

Chromatogram of SA-1 with Experimental Column #2
(Type I End-Capping)

a column that is not end-capped, a significant peak tailing is observed with minimal detectability for the new molecular probe at 0.1 µg level (Figure 2). With Type I end-capping at ~2% level, the peak tailing is reduced (Figure 3) and detectability is improved four-fold at 0.1 µg level. Type II end-capping at approximately the same level improved detectability eight-fold with a concomitant decrease in peak tailing and band-broadening (Figure 4).

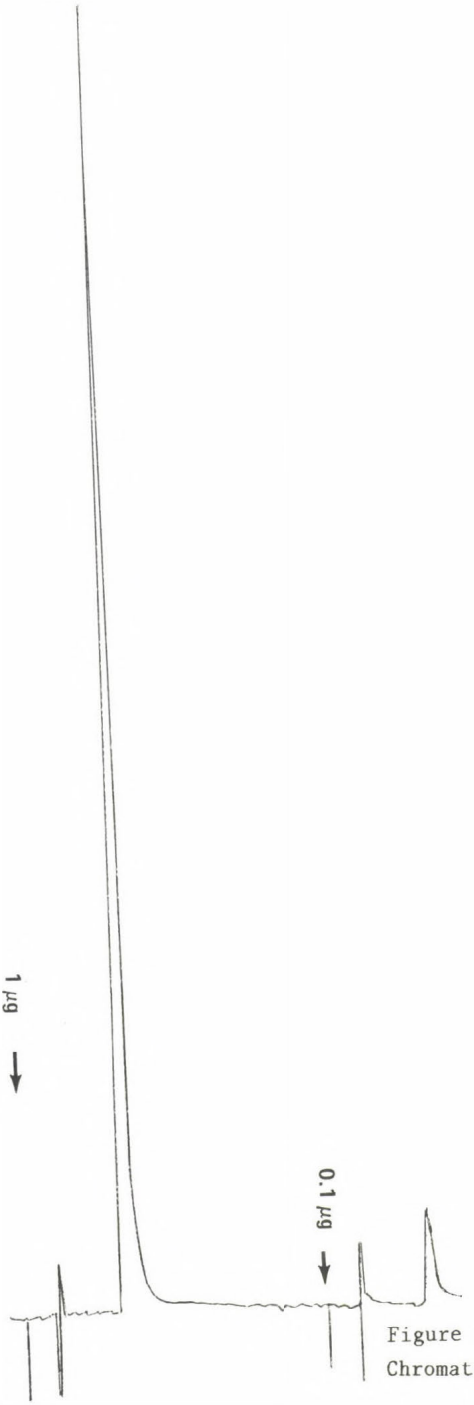


Figure 4

Chromatogram of SA-1 with Experimental Column #2
(Type II End-Capping)

CONCLUSIONS

To optimize selectivity and detectability, it is necessary to thoroughly understand stationary phase dynamics. Judicious use of select molecular probes is essential. Furthermore, to truly validate a method at ultratrace levels, it is desirable to specify the requirements such as peak width and height with a given probe at the submicrogram levels.

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SPACER-DISPLACEMENT THIN-LAYER CHROMATOGRAPHY

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SUMMARY

Conditions influencing displacement thin-layer chromatography are detailed.

Displacement developments employing column and planar arrangements of the stationary phase are compared, and column displacement chromatography is considered as a cyclic procedure. At the same time, displacement thin-layer chromatography is regarded as having steps which do not complete the whole cycle.

Results with displacement thin-layer chromatography suggest the use of preelution by the mobile phase at certain displacement procedure using HPLC or classical column chromatography.

The role of the spacer substance(s) in the improvement of displacement separations is discussed in detail.

INTRODUCTION

Based on the work of Tiselius (1), Porath et al. (2-4), Partridge (5, 6) and others, Horváth et al. renewed the displacement mode of development for preparative separation of samples employing high-performance liquid chromatography (7-10). The essence of their work can be summarized as the application of the theoretical basis of classical column liquid displacement chromatography to the conditions of high-performance liqpuid chromatography, and the utilization of the advantages of HPLC

for preparative scale separation with the instrumentation and columns generally used for analytical purposes.

Thin-layer displacement chromatography was also suggested by Horváth for scouting the optimum conditions of displacement chromatography of corticosteroids (11), to find the proper stationary and mobile phases, displacer and its concentration.

The planar arrangement of the stationary phase bed permits the simultaneous separation of several spots, or the use of two-dimensional development; the direct visual observation of the process how the displacement train is developed is also feasible. The essential advantage of thin-layer chromatography is the easy access of the separated spots on the sorbent layer. As the spots are on the surface (outer or internal), of the stationary phase, the localisation of the substances by color reagents and/or the detection of the activity of some spots is possible using autoradiography or bioautography, mentioning only the two most important methods where radiolabeled substances and radiolabeled compounds can be revealed. Naturally, the direct observation of the colored compounds is also provided by the planar arrangement of the stationary phase. This phenomenon inspired us to use colored substances (13) as spacers to separate the closely situated zones of the sample components and therefore improving the separation, facilitating the detection of the separated zones and giving a basically new concept for the evaluation of peak separation.

On the basis of our experiments using the planar arrangement of the stationary phase some characteristics of spacer displacement thin-layer chromatography (SD-TLC) will be described and discussed in this paper. The rules and discussion presented here may be, with certain limitations, also valid for column chromatography.

EXPERIMENTAL

Silica gel 60 F_{254} TLC plates, pre-coated on glass, 20 x 20 cm, were purchased from E. Merck (Darmstadt, FRG). Triethanolamine, methanol, and ethanol were purchased from

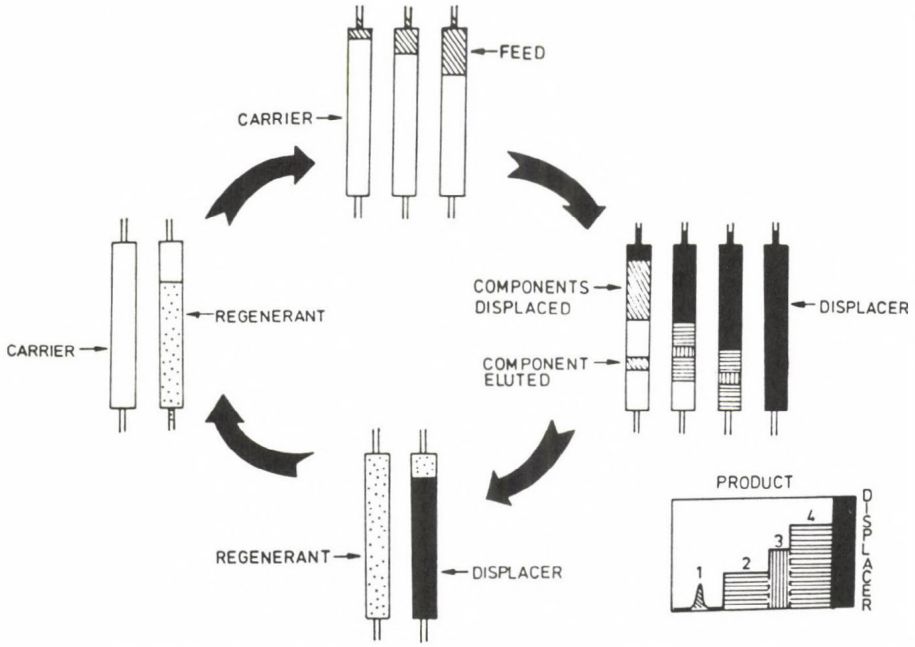

Fig. 1. The cyclic comprehension of displacement chromatography employing a stationary phase packed in a column

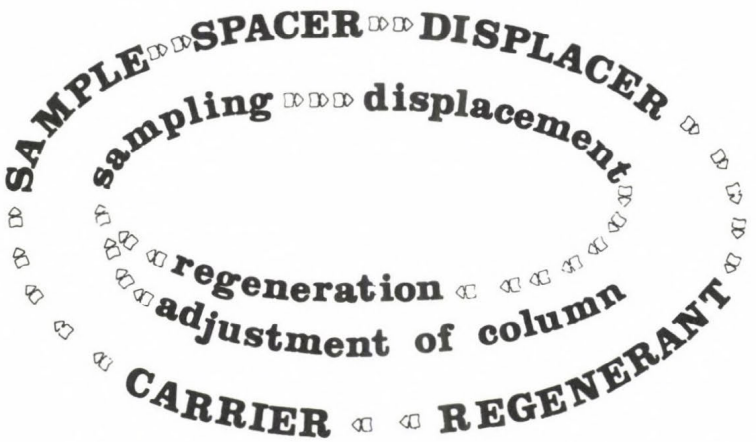

Fig. 2. Operational steps of column spacer-displacement chromatography starting with the adjustment of the column

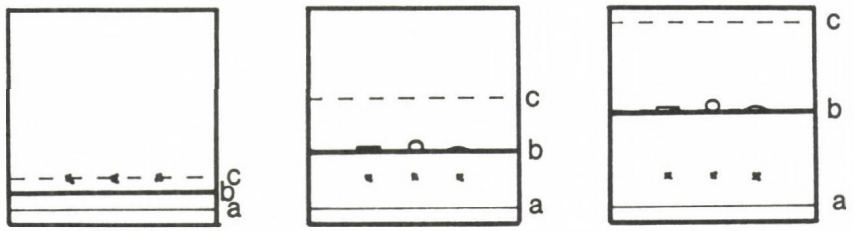

Fig. 3. Displacement thin-layer chromatography. The spotting of the samples is the subject of a separate procedure proceeding the step when the thin-layer plate is placed into the separation chamber

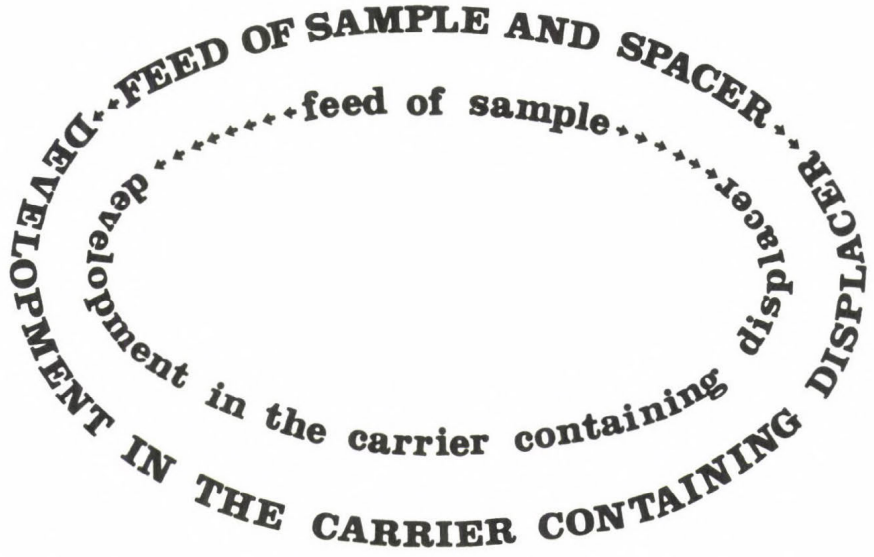

Fig. 4. Operational steps of spacer-displacement thin-layer chromatography

for the similar procedure in the following cycles of displacement chromatography may be "regeneration" (Fig. 2).

The situation is basically different in the case of thin-layer displacement chromatography. First of all, thin-layer plates are disposable, i.e., the regeneration can be omitted. The feed of sample is done in an independent procedure, and the remaining two operational steps, the feed of the mobile phase

Reanal (Budapest, Hungary). Deprenyl (N-methyl-N-propargyl-(2-phenyl-1-methyl) ethylammonium hydrochloride, JUMEX^{R}), propargylanara (N-propargyl-(2-phenyl-1-methyl) ethyl-ammonium hydrochloride), methamphetamine and amphetamine were the kind gift of Chinoin (Budapest, Hungary); benzoic acid and benzylamine were purchased from Fluka (Buchs, Switzerland). The dye-mixture marked as "Test Substance II" was kindly supplied by Dr.Jänchen (Camag Inc., Muttenz, Switzerland), while the displaced components of "Test Substance II" were marked as elements called "Sudanschwarz".

THEORETICAL

Principles of thin-layer displacement chromatography

In the case of displacement chromatography using a column prepacked with microparticulate particles, four consecutive steps of the operation were distinguished (7), namely when the feed solution, the displacer, the regenerant and the carrier are added into the column. However, these stages of the procedure cannot be handled separately; they are rather elements of an operational cycle, either for analytical or preparative separation (Fig. 1). Naturally, the order of the individual operational steps is strictly determined, but it should be simpler to handle them as the elements of a cycle. If any of the separation steps can be pointed out as the "first", it should be when the regenerant is fed. The subsequent stages are when the column is equilibrated with the mobile phase and when the sample is injected into the column. The final step consists of the actual displacement separation: the process when the development of the displacement train is in progress, the movement of the adjacent zones with the same speed up to the end of the column and when the exit of a product means the realization of the separation by fraction collection or detection of the progress of the product zones. However, the feed of regenerant and carrier in the very first cycle of the displacement operation should be called "column adjustment" while the term

and the displacer happen simultaneously, when the plate is developed in the mobile phase containing the adequate amount of the displacer. This scheme is illustrated in Figs. 3 and 4.

One more phenomenon (positional characteristic) differentiates high-performance displacement column and thin-layer chromatography. In the former the sample is injected at the front of the column where it is adsorbed at the inlet section. This means that the displacer will reach the sample to be displaced at the very first moment when it touches the column, i.e., just after the displacing development has been started. On the other hand, in the case of thin-layer displacement chromatography, the sample is dried on the TLC plate at a certain distance from its lower edge, and even at an adequate distance from the surface of the mobile phase containing the displacer. Therefore, a prerunning period of the displacer must be taken into consideration. This difference caused by the altering arrangements of the adsorption places on column and thin-layer displacement chromatography is demonstrated in Figs. 1 and 3.

Parallelism of elution and displacement chromatography

In any chromatographic process, the sample constituents are the objects of the separation. The basic condition of displacement chromatography is that the sample components are adsorbed at the inlet part of the column or at the adequate part of the TLC plate, while other portions are eluted. The situation was widely discussed in Horváth's paper (7) which pointed out that the operating line crosses the isotherms of the sample constitutents at the given conditions. If the adsorption isotherm of the component and the operating line has an intersection, the component will move by displacement; on the other hand, if they do not cross each other, the component will be eluted. When the concentration of the displacer in the mobile phase is increased, several other components (which did not have an intersection with the operating line, and therefore, were eluted) can be parts of the displacement train. On the other hand, if the concentration of the displacer is decreased, some earlier participants of the displacement train move faster

than the displacement front and they are eluted. This fact had been mentioned in the basic paper on high-performance displacement column chromatography; on the basis of our direct observations in thin-layer displacement chromatography, we can confirm this statement. However, the so called "preelution" in displacement TLC suggested us the possibility of a similar procedure in the case of displacement column chromatography, if the sample components to be isolated perform a very moderate movement in the mobile phase.

Improvement of the efficacy of separation by spacer substances

The zones of the displacement train were considered as consisting of three parts (7): the length of the plateau region, the length of the front and rear boundaries. The sum of these three parts corresponds to the whole length of the displaced zone. Therefore, the fraction of the i-th compound isolated in pure form can be given by the P value,

$$P = \frac{m_{i,t} - m_{i,f} - m_{i,r}}{m_{i.t}}$$

where $m_{i,t}$, $m_{i,f}$ and $m_{i,r}$ are the respective amounts of the i-th compound in the plateau, front and rear part of the zones.

In the case of spacer displacement chromatography, one or several zones are dividing the sample components to be separated. Therefore, the adsorption isotherms of the spacer substances as well as their zones should also be considered when the graphical representation of the isotherms and the concentration zones is arranged. Such presentation is given in Fig. 5, where the left side of the figure depicts the isotherm and the concentrations of the zones without the spacer, while the right side of the figure takes also the presence of the spacer into consideration. The concentration of the sample components and spacer zones in the displacement train are determined by the intersections of the operating line and the adsorption isotherms of the sample components and spacer substances. These intersec-

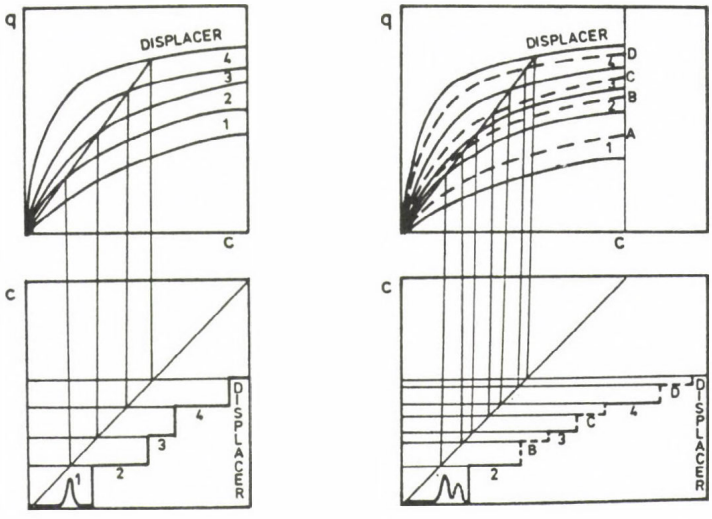

Fig. 5. Graphical representation of the adsorption isotherms and the concentrations of the separated zones. The left-hand side of the figure shows the separation without spacer substances, while the presence and role of the spacers are indicated in the right-hand side of the figure

tions give the height (concentration) of the zones while the length of the product zones are given by the total amounts of the individual components and by the concentrations of their zones.

While the presence of the spacer substances increases the total amount of the components present in the displacement train the extent of the overlap of the adjacent zones is practically eliminated: the overlap of the adjacent sample components is transformed into the overlap of one component zone and one spacer zone. At the same time, the spacer zone does not disturb the analytical separation of the sample component, and the spacer substances can be easily removed from the product. Therefore, the zones of the product can finally be obtained as individually appearing bands.

Influence of the spacer zones on the development of the displacement train

The progress of the development of the displacement train has not been considered in the earlier publications, and the question was simplified by stating that the "totally or fully developed displacement train" is completed. However, the generation of the fully developed displacement train has some requirements in both space and time. This space requirement depends on the quality of the components to be displaced and of the displacer as well as on the concentration of the displacer in the mobile phase. However, there is one more factor influencing the generation of the displacement train: the position of the zone

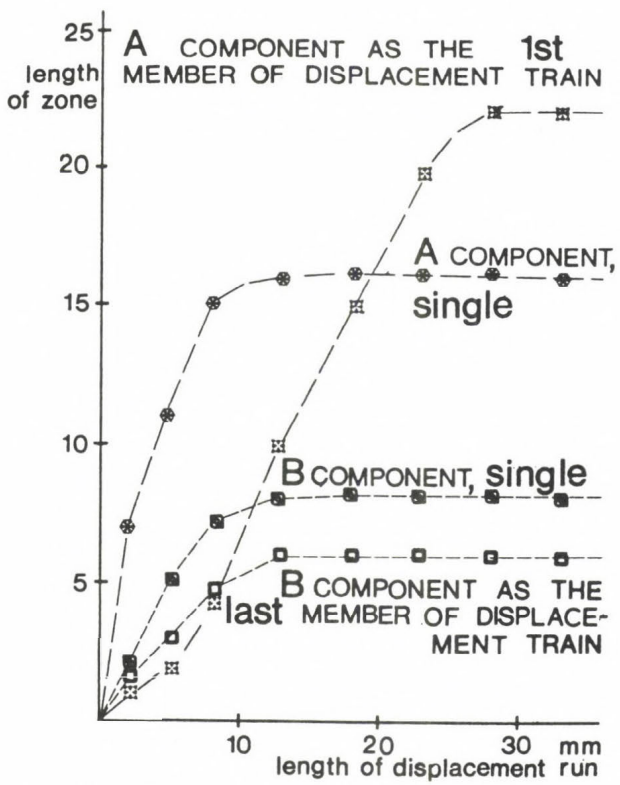

Fig. 6. The lengths of the displaced zones vs the lengths of displacement development give different curves depending on the position of the component in the displacement train

in the displacement train. This situation is illustrated in Fig. 6, where the length of the displacement zones is given versus the lengths of the displacement developments for two components in the case of two arrangements. If they are the only components of the displacement train, i.e., they are the single components in front of the displacer, or if they are the members of a multicomponent displacement train as the leading or terminating element, they behave differently. Fig. 6 clearly demonstrates that the single zone needs longer displacing procedure for its full development but the length of such a zone may be longer. On the contrary, the terminating zone requires only a very short length of displacement but the length of the zone is compressed by the common effect of the displacer and the displaced zones preceding the particular compound.

RESULTS AND DISCUSSION

Spacer displacement thin-layer chromatography of deprenyl and phenylethylamine is shown in Fig. 7. The samples were spotted at different distances from the bottom edge of the thin-layer plate; therefore, after completing the development, they were also moved for different distances in the displacement train. The displacement distance under a certain value is not enough to fulfill the requirement of the generation of the totally developed displacement train. Furthermore, if the fully developed displacement train has been generated, the lengths and the relative position of the zones do not change any more.

Displacement thin-layer chromatography can be applied in combination with elution type of development. Fig. 8 shows the separation of several components using elution type development in the first dimensional run and displacement type development in the second dimension. After the elution type development had been completed, the plate was dried and the spacer substance, in this case Sudanschwarz, was lined in the start of the second-dimensional development. All substances investigated here were displaced and the black lines of Sudanschwarz surrounded the components thereby highly facilitating both the improvement of

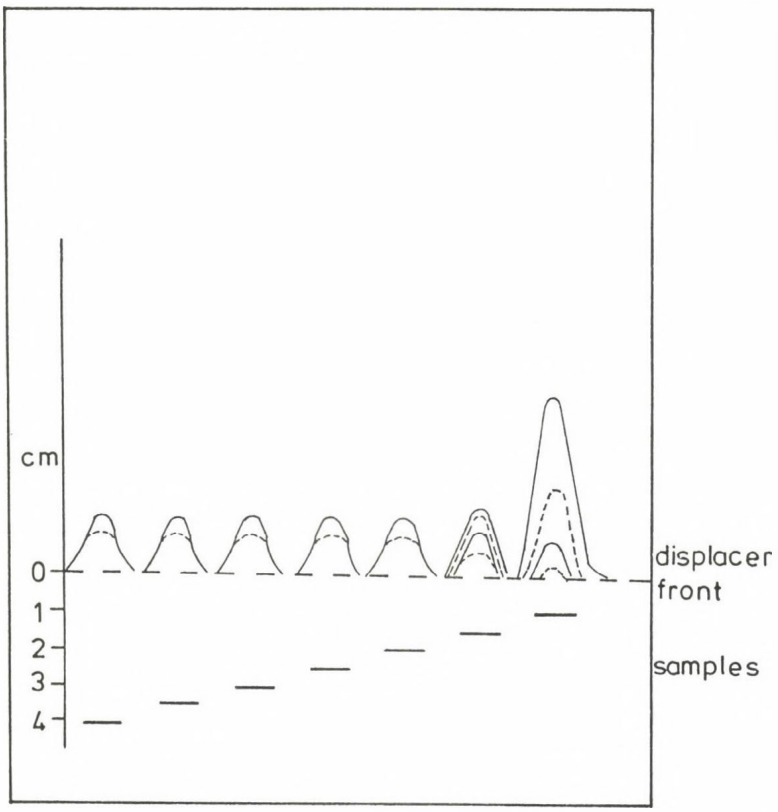

Fig. 7. This illustration presents how the displacement train is generated. The displacement train was generated for 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 cm; any distance which is at least 2.0 cm is enough for the fully developed displacement train

separation and the direct visual observation of the separation. Using a certain preelution with the mobile phase, one of the spots can be removed from the displacement train, decreasing thereby the "spot traffic jam" in front of the displacer.

As the basis of the separation is not only displacement chromatography but the subsequent application of elution and displacement developments, it is very important that the separation power of both types of chromatography should be fully exploited. Such type of optimization is demonstrated in Fig. 9

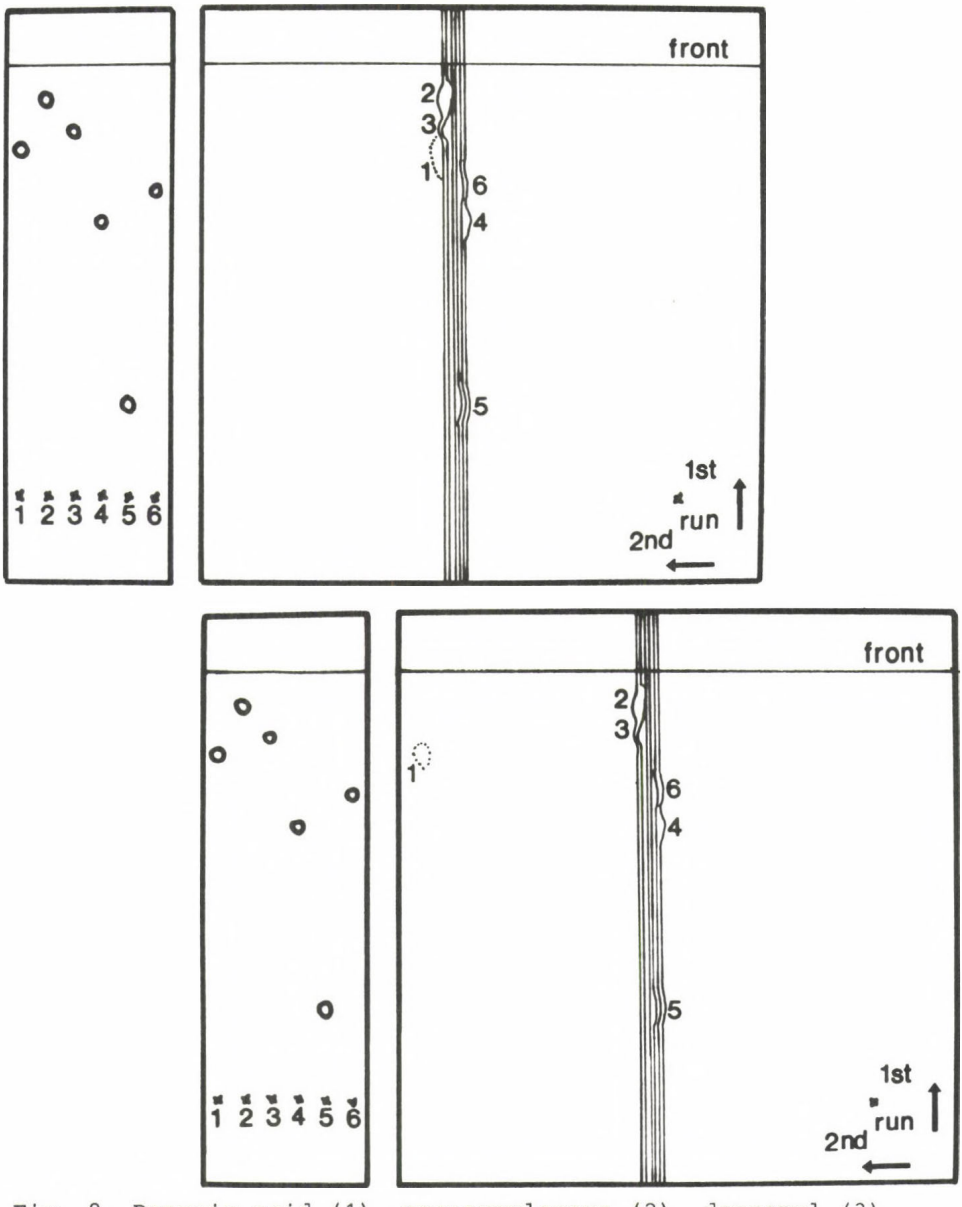

Fig. 8. Benzoic acid (1), propargylanara (2), deprenyl (3), N-methyl-phenyl-ethyl-amine (4), amphetamine (5) and methamphetamine (6) are separated by two dimensional (elutioncarrier displacement) thin-layer chromatography without and with preelution by the mobile phase in the second dimensional run. The first and second dimensional developments were performed in 7:5:1 chloroform-methanol-phosphate buffer (pH 6) and 95:5 chloroform-triethanolamine, respectively. Preelution was carried out with chloroform mobile phase

where 0.5, 1.0, 2.5 and 5.0 mg of both deprenyl and propargyl-anara were separated; the best separation can be found when 1.0 mg of each substance is spotted. This point of view was applied when radiolabeled metabolites were separated using inactive standard compounds as markers and spacer substances (12).

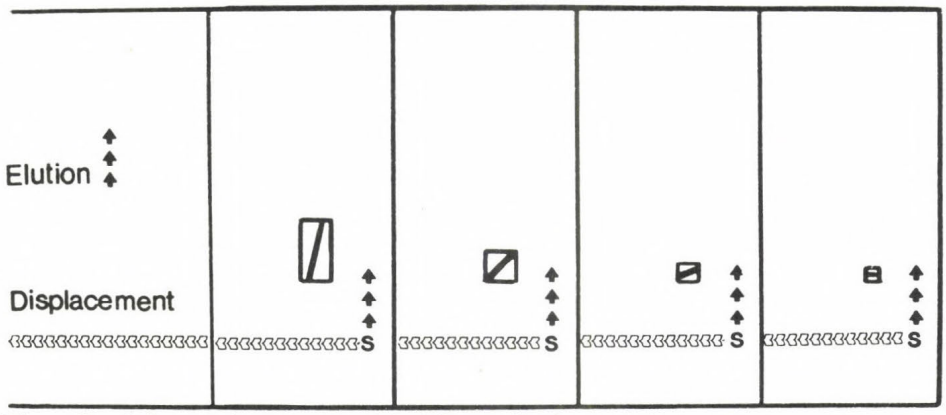

Fig. 9. Schematic representation of the optimization possibility in elution - displacement two dimensional TLC

CONCLUSIONS

Displacement development consists of several individual steps when the regenerant, the mobile phase, the sample and the displacer are fed into the separation system. These individual steps should be regarded as parts of the whole cycle. Any step except displacement can be the first member of the cycle which should be preceded by the feed of the sample. However, the adjustment of the column (the feed of regenerant and the mobile phase) can be distinguished as the first procedure and the other steps total the cycle.

Displacement thin-layer chromatography (DTLC) and high-performance displacement column chromatography (HPDC) differ from each other in two main respects. In DTLC, the regeneration step is neglected because separation takes place on disposable plates. In addition, the stationary phase is preequilibrated with the mobile phase when the samples have been spotted on the plate. Therefore, DTLC is realized by a single procedure, when

the bottom edge of the TLC plate is dipped into the mobile phase solution containing the displacer, and the simple run gives both development in the mobile phase and the movement of the displacer. On the basis of the DTLC experiments, two basic conclusions can be drawn:

a) *The location of the sample components, after their feed was introduced but before the displacer is fed, is not uncondi - tionally the fore part of the chromatographic stationary phase. They can move with slow but essential speed along the chromatographic system. If this movement is slower than the flow velocity of the displacer front, the procedure can be regarded as displacement; on the other hand, if the speed of their movement is faster than that of the displacer front, the substances are eluted by the mobile phase. This phenom- enon is clearly demonstrated by the graphical representation of the adsorption isotherms and the operating line: the latter may or may not cross any of the adsorption isotherms, depending on the slope of the operating line which is de- pendent on the concentration of the displacer (7). However, not the adsorption of the displaceable components but their movement with a limited speed should be the basis of displa- cement chromatography.*

b) *If the component to be isolated has an intermediate position among the members of the displacement train, the adequate choice of the displacer concentration can help in removing a part of the contaminating substances which locate before the component in question. In this case, either preelution with mobile phase, or gradient elution (stepwise or continuous) with the displacer or a properly selected concentration of the displacer can help in removing the majority of the con- taminants, preceding the substance to be isolated before the actual displacement chromatography has been completed. The same result can be reached by using a precolumn between the pump and the sampling device.*

In the case of DTLC, the direct visual observation of the displacing process itself as well as of the separation of the sample components by the displacement train can highly facilitate the reliable separations. Additional advantage of DTLC is the possibility of the so-called "contact" evaluation methods including the use of color reagents, autoradiography or bio-autography (11-16).

Two-dimensional thin-layer chromatographic separation has realized an elution-type development in the first dimension and displacement-type development in the second dimension. The optimization procedure includes the utilization of development in both directional runs.

The presence of spacer substance(s) can cause a slight alteration in the parameters of the fully developed displacement train including the lengths of some zones and the distances which are required for the total development of the displacement train. However, the major effects of the spacer substances are that they can highly facilitate the direct visual observation of the separations; also, spacers make displacement separation possible without any overlap of the sample components.

As the spacer substance(s) can be removed after the separation is completed, the series of the zones emerging at the end of the column can be considered as distinct zones without any overlapping. However, this situation should also be maintained after fractionation. For this reason, the amount of the spacer has to be selected so that the spacer zone should be wider than the volume (or time) of fractionation. In this way the collected fractions will not show any cross-contamination.

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APPLICATION OF ION CHROMATOGRAPHIC METHOD IN ENVIRONMENTAL STUDIES

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SUMMARY

Liquid chromatography was successfully used for the routine analysis of inorganic anions in natural waters. Minimum detectable quantities were 10 ppb for F^- and Cl^-, 50 ppb for NO_2^- and NO_3^- and 0.2 ppm for PO_4^{3-} and SO_4^{2-}. The standard deviation was 1.5%.

Liquid chromatography was used in biogeochemical monitoring and some results of the investigations are presented. Certain problems of migration of chemical elements in natural water circulation processes and ecological indication are solved.

INTRODUCTION

Industrial pollution along with emissions from natural sources can affect long-term changes in the biosphere. The diagnosis of the damage of environmental pollution to the biosphere is not easy because it can occur in many different forms. First, the interactions between the pollutants and the components of biosphere have to be established. As the geochemical transfer of the individual chemical elements (including pollutants) takes mainly place in the hydrosphere and the atmosphere the routine methods are needed for the analysis of water and air samples in order to determine the distribution of the pollutants in nature.

Considering the specific character of the problem in question at least three features are essential in the selection of the analytical technique. First, as every geochemical process in result represents the statistical transfer of certain chemical elements the analytical equipment must permit a large number of routine determination. Second, it is necessary to simultaneously detect the large number of different chemical substances present. Third, since chemical elements are present in a wide concentration range in natural samples, the analytical instrument must have a wide dynamic linear range for every element (substance) analyzed.

Detection methods based on the measurement of changes in the electrical conductivity have been found to be suitable for the detection of chromatographically separated ionized chemical substances /1-3/. The application of conductometric detection in ion-exchange technique succeeded when the problem of suppressing the background conductivity caused by the eluting electrolyte was solved /4/.

In recent years ion chromatography has found more and more use as a universal tool for environmental monitoring, specially for the analysis of waste streams, rivers, lakes, precipitation and even fluids of biological interests /5/. Therefore it is understandable why so many companies (Dionex, Biotronik, Wescan, etc.) are busy in designing and producing ion chromatographs. This is the reason why the problem has also caught our attention.

The idea to use a conventional liquid chromatograph in conjunction with a conductivity detector to convert the liquid chromatograph into an ion chromatograph is not new but it is a good approach. It significantly increases the value of the system once obtained.

EXPERIMENTAL

Chromatographic equipment

The system used consists of the conventional liquid chromatograph Model AVK-31 and conductivity detector Model JD-1,

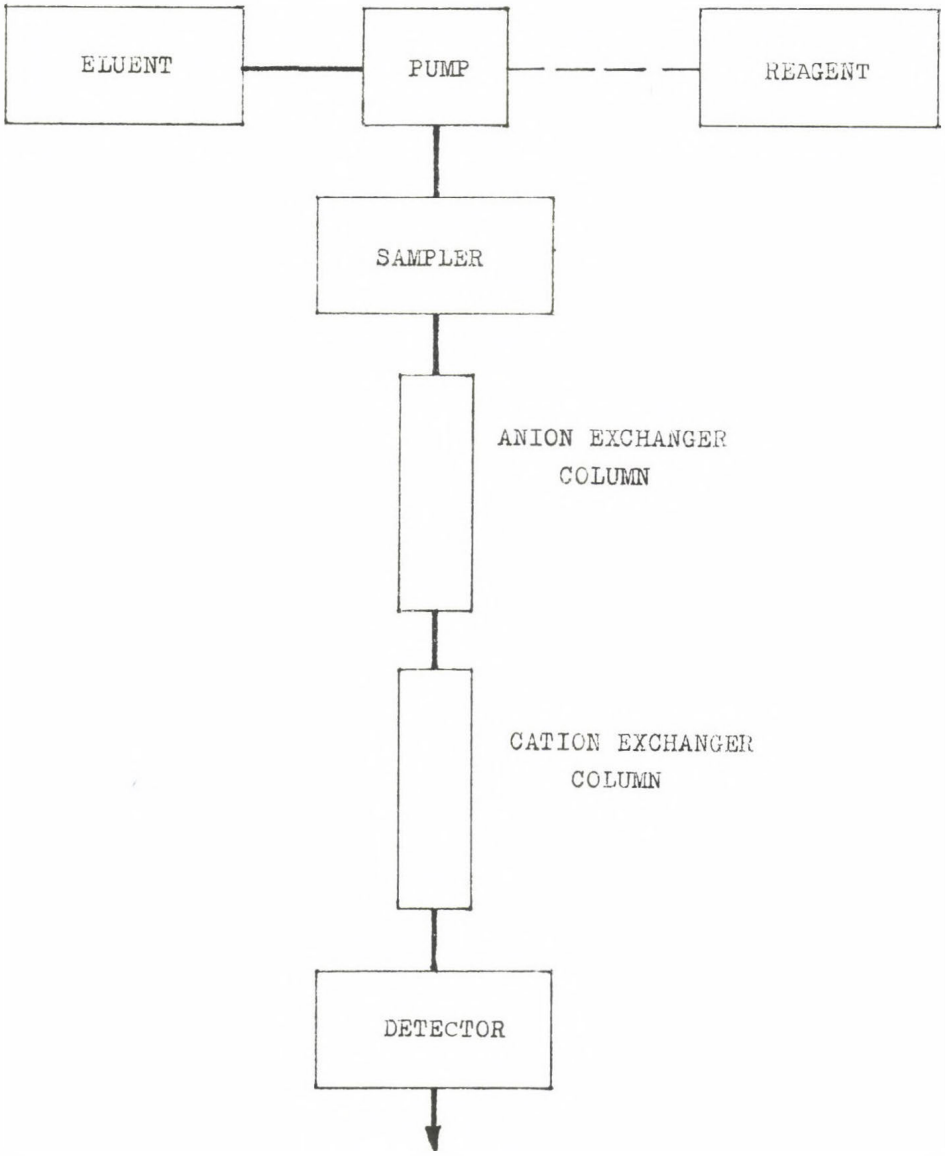


Fig. 1. Block diagram of the chromatographic system

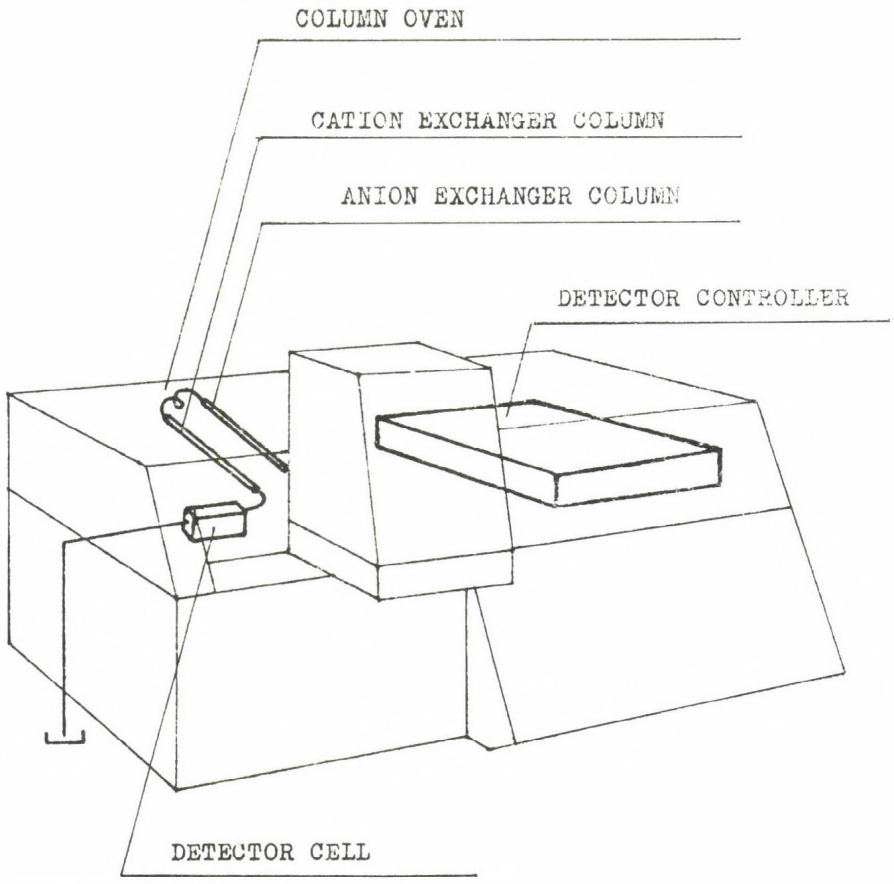


Fig. 2. Schematic diagram of the installation of the conductivity detector JD-I and the columns into the Model AVK-31 liquid chromatograph

both designed and produced by the Special Design Office of the Academy of Sciences of the Estonian SSR.

The block diagram of the system is shown in Fig. 1. A reciprocating pump operating under constant volume conditions was used for pulseless solvent delivering for separation and regeneration. Direct on-column sample introduction was achieved with a novel automatic sampler for sample volumes in the range of 2 to 20 µl. For larger sample volumes a loop injector was used.

The JD-1 detector Model represents an alternative current conductivity detector at the frequency of 10 kHz. Measurement voltage is 0.5 V. Cell volume is about 2 µl. The range of measurable conductivities extends to 10^{-4} S.

The detector was mounted in the column oven (Fig. 2). Although the AVK-31 chromatograph Model has an excellent thermostat (temperature stability better than $\pm$ 0.1°C) the detector had an additional temperature compensator. Full scale zero suppression allows to use eluents with different background conductivities.

As the basic characteristics of the liquid chromatograph AVK-31 have been published /6/ only the characteristics of the Model JD-1 conductivity detector are given here in more detail.

The detector cell constant, P, was determined by measuring the conductivity of a 0.0005 N KCl solution at 18°C. P was found to be 3 cm^{-1}.

The almost pulseless, constant-flow pump guaranteed the baseline stability. The noise was typically less than 1% and the drift did not exceed 10%/hr at the most sensitive range.

Minimum detectable conductance change was 10^{-4} µS.

Resolution of the detector can be expressed by the concentration of a KCl solution flowing through the cell which causes the detector response to drift 1 mV at the output. Resolution at 18°C was found to be 3.7×10^{-8} g KCl/ml. mV. The response of the detector cell was linear up to 0.005 N KCl solution.

A 250 x 4.0 mm separation column and a 150 x 6.0 mm suppressor column were used.

The anion exchange resin with a particle diameter of 0.025-0.040 mm and a capacity of about 12.7 µeq/cm^3 dry material was synthesized by the Institute of Chemistry, Academy of Sciences of the Estonian SSR. In the suppressor column the cation exchange resin KY-2 /7/ with a capacity of about 3.0-4.9 mg.eq. g^{-1} dry resin was used.

The eluent was prepared by dissolving 0.126 g $NaHCO_3$, 0.127 g Na_2CO_3 and 0.120 g NaOH in 2 litres of bidistilled water.

RESULTS AND DISCUSSION

Application of the ion chromatograph, just as the application of every conventional liquid chromatograph needs the special calibration of the system.

It is known /8/ that the calibration plot in ion-chromatography with eluent suppression is generally not linear regardless whether peak heights or peak areas are used. The main reason for this is that in the eluate from the suppressor column the dissociation of the eluent is suppressed by the H^+-ions from the sample, if the eluent is weakly acidic and the sample is strongly acidic. Therefore, the contribution of the eluent to total conductivity is not constant.

For the investigation of the characteristics of our system calibration was carried out at different conditions. The results of our investigations are given in Fig. 3. Samples with Cl^- concentrations ranging from 3.53 to 200 mg/l were injected with a 20 µl and 100 µl loop. The calibration plot between sample concentration (or quantity of Cl^-) and peak area (or height) consists of two linear curves with different slopes. The so-called critical Cl^- concentration where the slope changes is of about 50 mg/l for the 20 µl loop when using the peak area and about 25 mg/l when using the peak height. As shown in Fig. 3, the value of the critical concentration does not depend on the eluent flow rate but depends on the injected sample volume as different amounts of Cl^- were injected. Calibration of Cl^- based on the integration of the amount of Cl^- over the peak area shows no significant dependence on the sample volume. The critical amount of Cl^- in the sample was about 1 µg. For the routine analysis it is useful to know that if the amount of Cl^- in the sample exceeds 1 µg a smaller sample volume has to be injected or the sample has to be diluted.

As shown by our investigations (Figs 3, 4) there is a dependence of system response on the eluent flow rate. The dependence was tested by using five different flow rates in the range of 0.5-3.0 ml/min and sample volumes of 20, 100 and 200 µl of 0.001, 0.0002 and 0.0001 N KCl solutions, respectively. As shown by our results the product of the peak area and the eluent

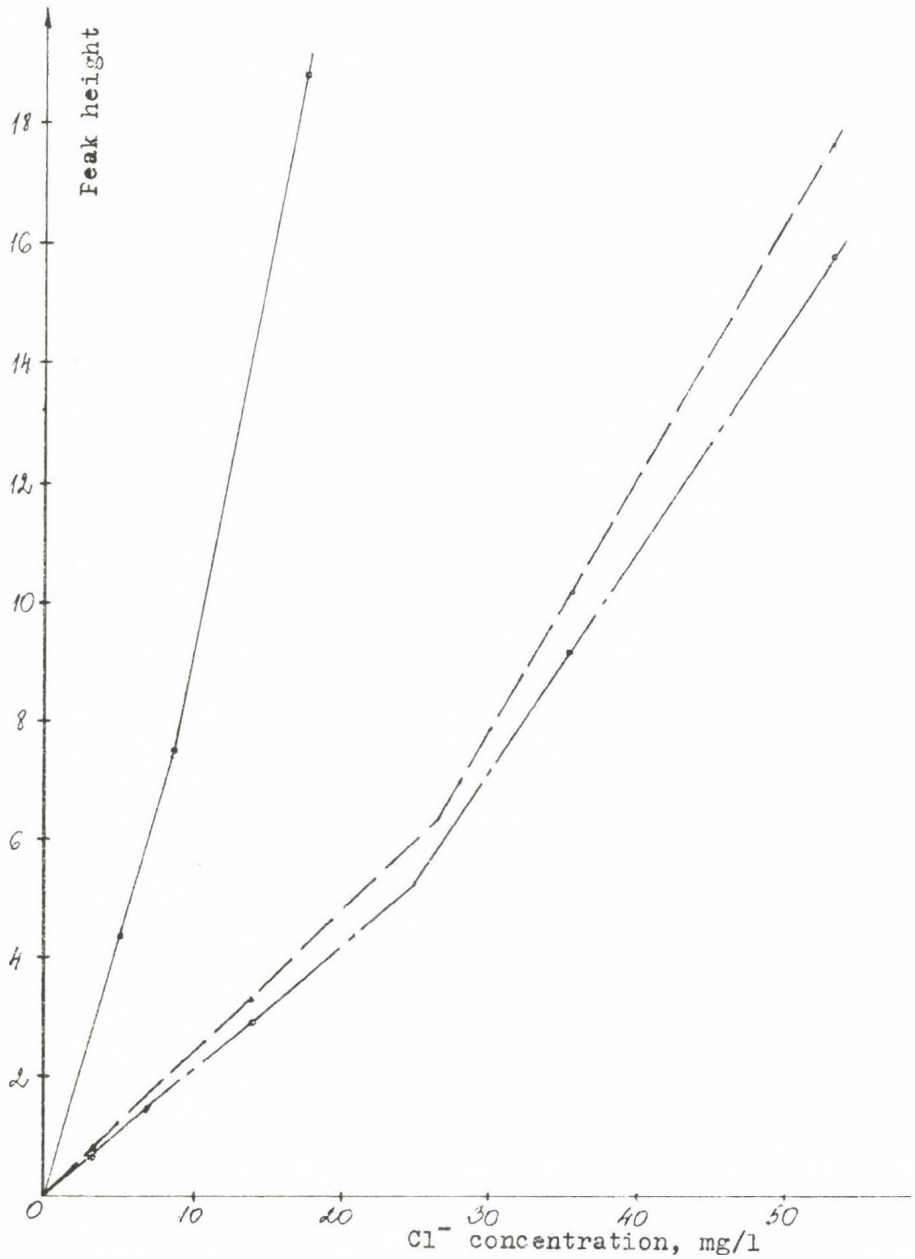


Fig. 3. Calibration graphs for Cl^- at different eluent flow rates for 20 and 100 µl loops (peak height version)
— — — 1.5 ml/min, 20 µl loop
—— · —— 2.5 ml/min, 20 µl loop
———— 2.0 ml/min,100 µl loop

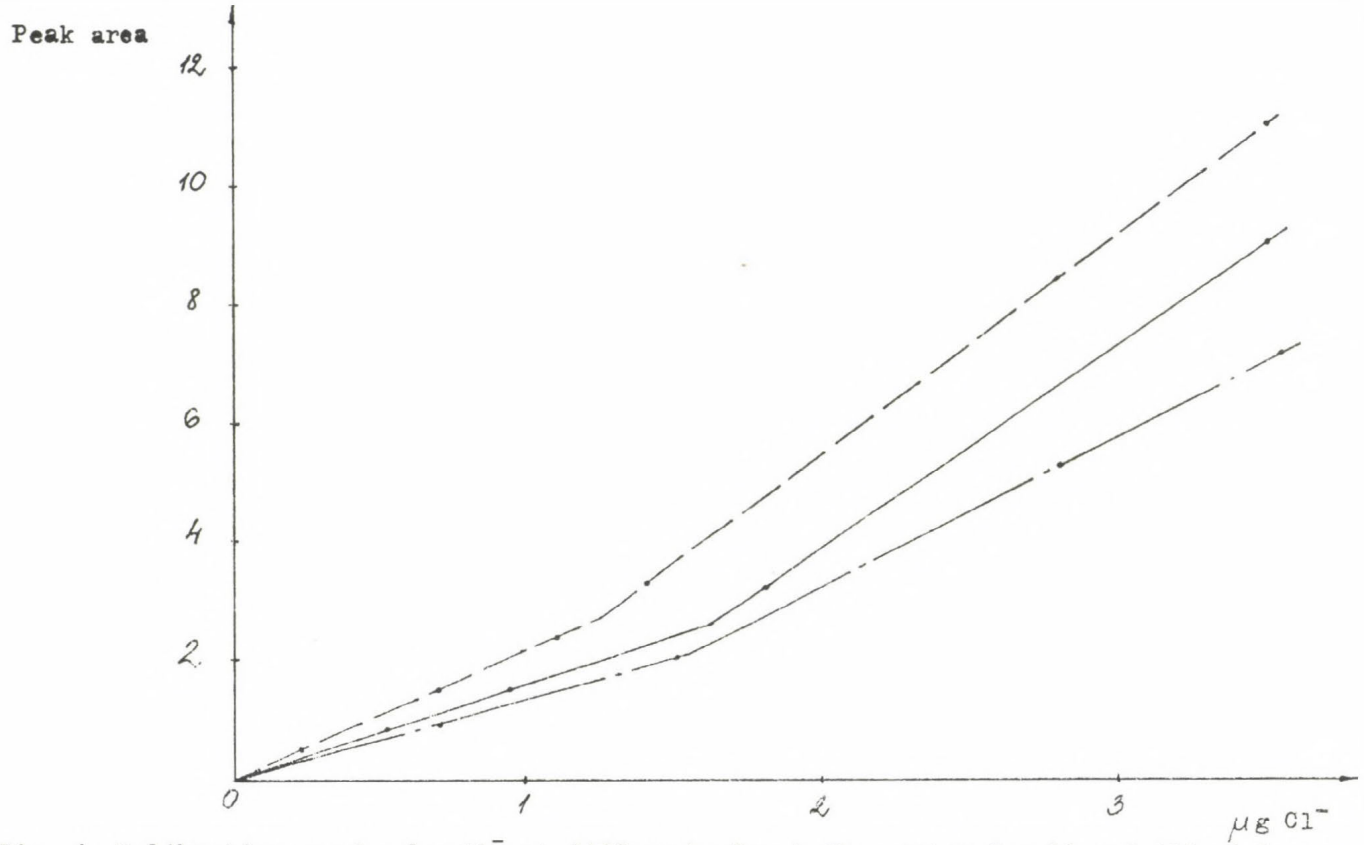


Fig. 4. Calibration graphs for Cl^- at different eluent flow rates for 20 and 100 µl loops (peak area version)

—— · —— · —— 2.5 ml/min, 20 µl loop
—— —— —— —— 1.5 ml/min, 20 µl loop
———————— 2.0 ml/min,100 µl loop

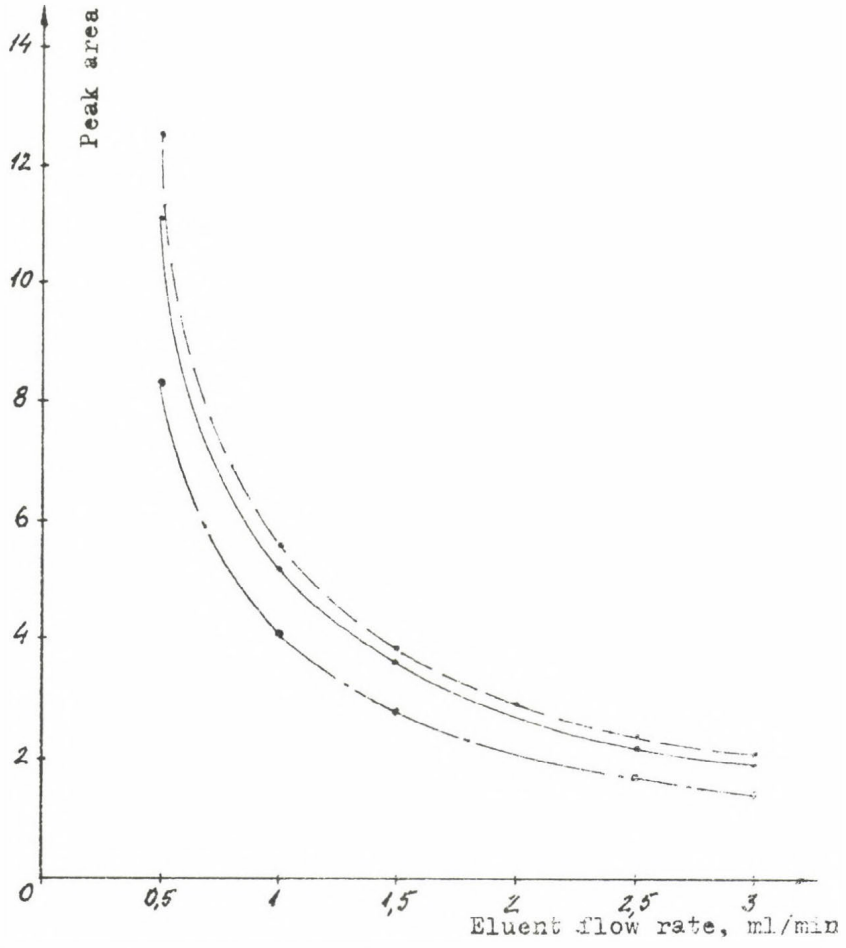


Fig. 5. Influence of the eluent flow rate on the peak area at constant Cl^- concentration in the injected sample (710 ng Cl^- in the sample)

— . — . _ sample volume 20 µl

— — — sample volume 100 µl

———— sample volume 200 µl

flow rate remains constant within the range. It must be pointed out that at eluent flow rates less than 1.0 ml/min a change of 0.1 ml/min in the flow rate causes a change of about 20% in the peak area (at the same sample concentration) while its influence to the peak height is about 3% (Fig. 5). Therefore, for quan-

titative analysis the use of the peak-height is recommended in order to diminish the possible impact of an unstable eluent flow rate.

One of the most important characteristics of chromatographs is the minimum detectable quantity, Q_o, defined as the concentration which gives a detector response equal to twice the noise level. Due to the low noise-level the minimum detectable quantities for different species are as follows: 10 ppb for F^- and Cl^-, 50 ppb for NO_2^- and NO_3^-, 0.2 ppm for PO_4^{3-} and SO_4^{2-}.

The minimum detectable quantities of different species were determined by injecting a 20 µl volume of a standard mixture (4 mg/l F^-, 5 mg/l Cl^-, 20 mg/l NO_3^- and SO_4^{2-}, 40 mg/l NO_2^-, 100 mg/l PO_4^{3-}). The eluent flow rate was 2.5 ml/min. Decreasing the eluent flow rate the minimum detectable quantities also decrease: for example at an eluent flow rate of 1.5 ml/min Q_o is 0.5 ppb for chloride, using the 20 µl loop.

The relative standard deviation for ten runs of 20 µl 0.005 N KCl at an eluent flow rate of 2.5 ml/min using the peak height was 1.5%, but for the peak area it was found to be 3.5%.

For practical use it is recommended to determine the capacity factor of the system. Capacity factor data for chloride were calculated with the equation $k' = (t - t_o)/t_o$ /7/ where t and t_o are the retention times of the retarded component and water, respectively. The k' values, listed in Table 1, were determined by injecting 20, 100 and 200 µl of 0.001, 0.0002 and 0.0001 N KCl solutions, respectively, at different eluent flow rates.

Table 1. Capacity factors for chloride

Eluent flow rate, ml/min	k'		
	Sample volume		
	20 µl	100 µl	200 µl
0.5	0.46	0.42	0.40
1.0	0.44	0.40	0.38
1.5	0.44	0.46	0.35
2.0	0.44	0.40	-
2.5	0.38	0.35	0.31

The concentration of different species in natural samples may change in a very large scale. Therefore, frequent switching of ranges is necessary. This is performed manually. For quantitative analysis the dilution of the samples is often needed. However, for the determination of trace components the natural sample should be analyzed first without diluton.

Applications

Ion chromatography can be applied to determine the background levels of some compounds in natural objects and tne changes of these levels under the influence of natural processes and human emissions. Considering the specific character of ion chromatography the method finds primarily wide use in the analysis of natural waters. A great part of our analyses are connected with the determination of the chemical composition of waters at different stages of natural cycling, from atmospheric precipitations to the surface water of industrialized regions. Regular investigation of trace impurities in precipitations enables us to establish the variations in the ionic composition of the precipitation from the natural conditions and evaluate the human influence, as well as to identify the pollution sources and investigate the dynamics of the deposition of the pollutants. Of main importance here is the great sensitivity of the analytical system and the reproducibility of the results. Fig. 6 presents concentration data of different species in the average monthly precipitation in an industrialized district. By the analysis of the ionic composition of surface waters it is relatively easy to find the pollution source and to follow the migration of impurities from the sources up into the purification system,and investigate the efficiency of purification, etc. The ion chromatograph seems to be a hopeful tool for the investigation of the impurities from atmospheric precipitations in the polar region as well as of relatively contaminated surface water from intensive industrial and agricultural areas.

The most exciting application of ion chromatography is to study the relationship between airborne compounds, pine bark chemistry and pine-inhabiting lichens.

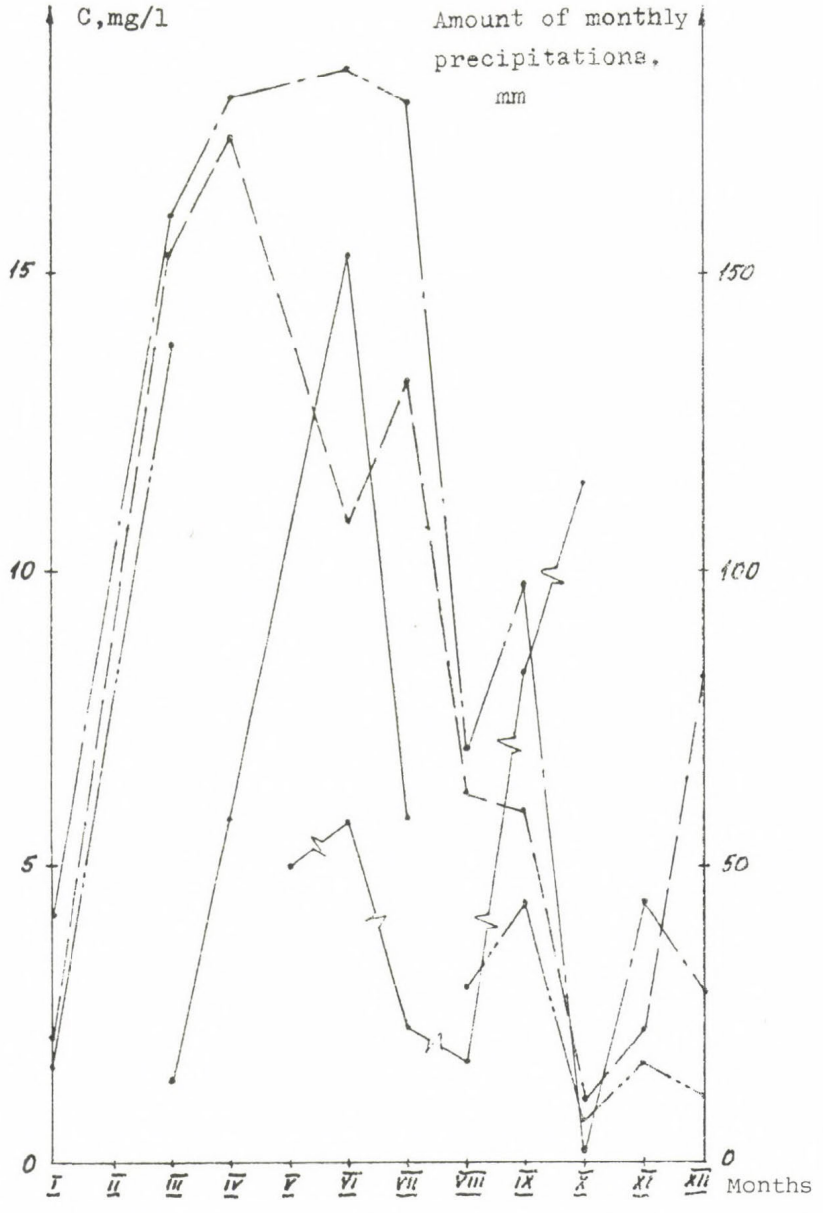


Fig. 6. Concentration of different anions in average monthly precipitations

— — — SO_4^{2-} — .. — Cl^-

— . — NO_3^- ———— F^-

—∿— amount of monthly precipitations

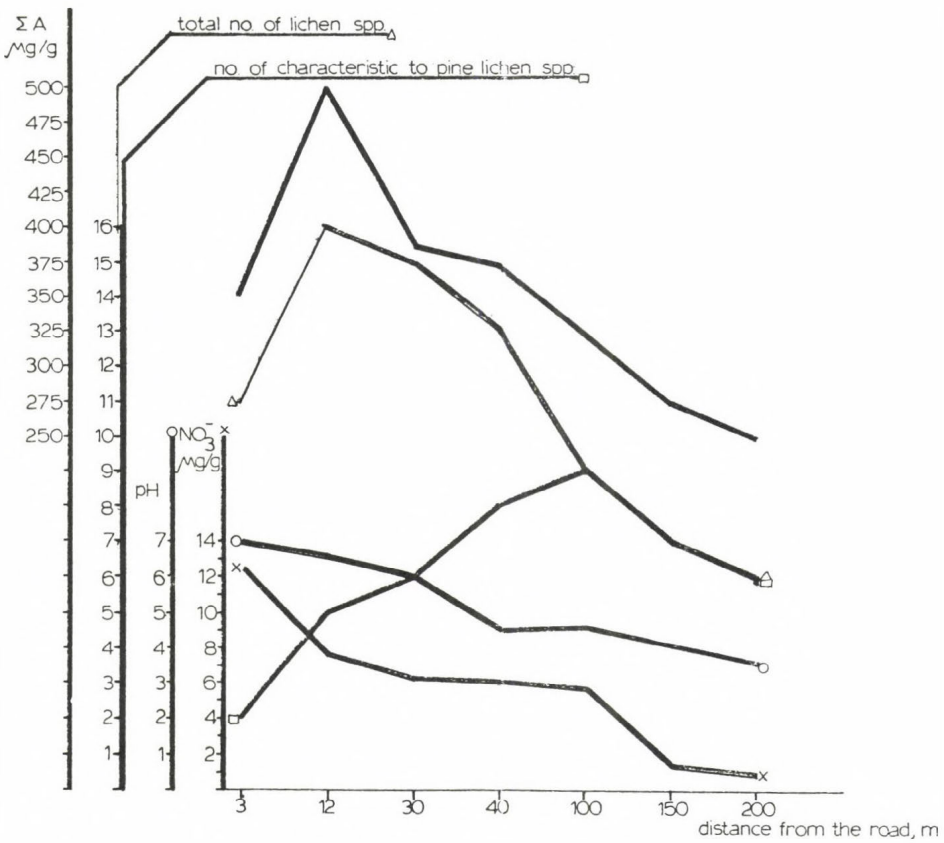
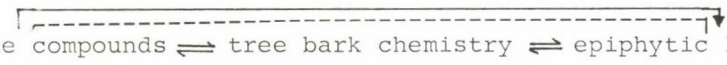


Fig. 7. Influence of road dust on the epiphytic lichen vegetation of pine trees

Tree bark can accumulate considerable amounts of airborne compounds and, accordingly, change its chemistry. At the same time, the species composition of the tree-inhabiting lichen vegetation depends closely on the bark chemistry as well as directly on the air quality. The lichens themselves can accumulate even more substantial amounts of airborne compounds. The case may be presented by the following scheme:



where the solid lines show the direct influence and the dotted lines - the possibilities of ecological indication. Ion chromatography can be applied to every unit of the scheme to solve the problems of lichen ecology and to be a method for biogeochemical monitoring of environmental quality.

Fig. 7 demonstrates the influence of dust pollution from a gravel-covered road. The analyzed pine bark was collected and epiphytic lichen species were determined on the transect moving away from the road. Near the road the lichens were represented mainly by the species of subneutral nutrient-rich bark which in natural conditions are characteristic of broadleaved trees. With growing distance from the road their proportion decreased and correspondingly increased the role of lichen species characteristic to acidic nutrient-poor pine bark in natural conditions. At a certain distance only the characteristic to pine lichen species have remained. So, the lichen species are dependent on the bark chemistry and can indicate its changes. The graph shows good accordance of lichen species composition and number with the bark NO_3^- - and sum of anions of water-soluble compounds ($\Sigma A = F^- + Cl^- + NO_3^- + PO_4^{3-} + SO_4^{2-}$) content (µg/g), determined by ion chromatograph, and the bark pH.

Fig. 8 shows the influence of some airborne compounds carried by the dominating western winds on the pine bark chemical composition (anions of water-soluble compounds) on a West-Estonian island.

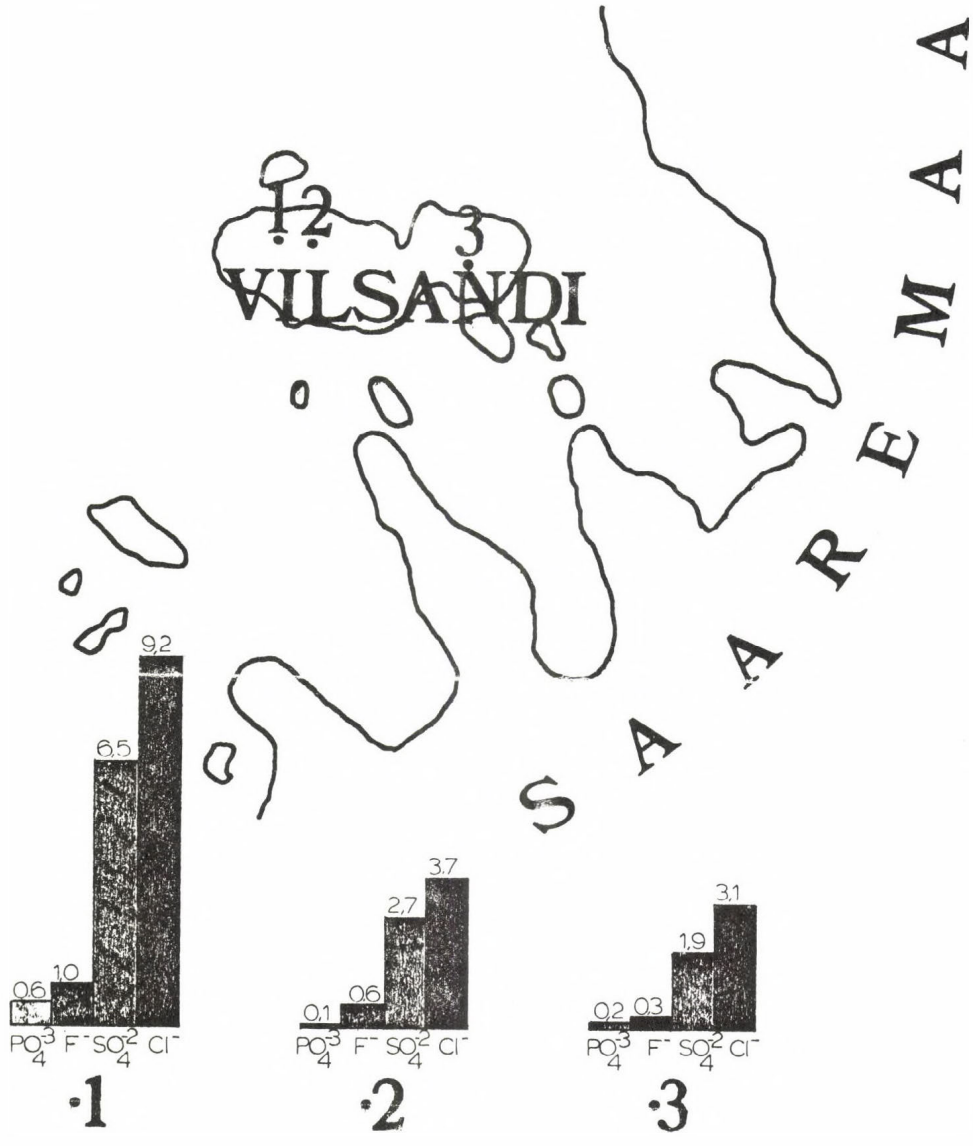


Fig. 8. Influence of some airborne compounds carried by predominant winds on pine bark chemistry (anion µeq/g of bark)

CONCLUSIONS

The analytical liquid chromatograph Model AVK-31 equipped with the Model JD-1 conductivity detector represents a universal tool for enviromental monitoring. The system has high sensitivity, good reproducibility and may be used in a wide concentration range. For the calibration of the system the dependence of the peak height on concentration at fixed eluent flow rate must be determined.

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H. Kalász and L.S. Ettre (Eds)

HOW TO FIND A CHROMATOGRAPHIC PARTITION SYSTEM FOR CHARACTERIZING THE 1-OCTANOL/WATER PARTITION?

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SUMMARY

It is shown that the relationship between the reversed-phase chromatographic capacity factors (k') and the 1-octanol/water partition coefficients ($K_{o/w}$) referring to structurally unrelated compounds is influenced by the organic phase concentration of the eluent in which the k' values were determined. A method is suggested to find an optimum organic phase concentration of the eluent at which the log k' values show the closest correlation to the log $K_{o/w}$ values. Using Supelcosil LC-18 and LiChrosorb RP-18 stationary phases the best relationship between log k' and log $K_{o/w}$ values was found when about 30% (v/v) acetonitrile was used as the eluent in both cases.

INTRODUCTION

Hydrophobic properties of drugs play an important role in their pharmacological action, as they govern adsorption processes and receptor binding /1/. The distribution pattern of organic contaminants, the bioaccumulation potential and the toxicity of organic compounds are also related to their hydrophobic character /2-6/.

Hydrophobicity of compounds is usually characterized by the logarithm of the 1-octanol/water partition coefficient (log $K_{o/w}$). The 1-octanol/water partition system proved to be a good model for characterizing the partition processes in

Table 1. The $\underline{m}$ and log k'_o values of different compounds calculated according to eq. 4 from the measured retention data on Supelcosil LC-18 and LiChrosorb RP-18 columns*

Compound	Supelcosil LC-18		LiChrosorb RP-18		log $K_{o/w}$*
	$\underline{m}$	log k'_o	$\underline{m}$	log k'_o	
Sulfadimidine	-0.0566	1.362	-0.0280	0.854	0.32
Nicotinamide	-0.1041	0.739	-0.0382	0.251	-0.57
Acetoazolamide	-0.0679	0.987	-0.0531	0.765	-0.26
Sulfamethoxy-pyridazine	-0.0680	1.631	-0.0285	0.892	0.40
Caffeine	-0.0876	1.590	-0.0299	0.552	-0.07
Chloramphenicol	-0.0536	1.889	-0.0414	1.625	-1.14
Salicylamide	-0.0413	1.227	-0.0255	0.871	1.28
Phenacetine	-0.0348	1.408	-0.0226	1.002	1.58
Methylsalicylate	-0.0398	2.571	-0.0244	1.727	2.46
Acetanilide	-0.0369	1.189	-0.0270	1.021	1.16
Benzaldehyde	-0.0195	1.028	-0.0303	1.575	1.45

*The log $K_{o/w}$ values were taken from the compilation of Hansch and Leo /18/

LiChrosorb RP-18 column:

$$\log K_{o/w} = 45.24\ \underline{m} + 1.501 \log k'_o + 0.724 \qquad (7)$$

$n = 11 \qquad r = 0.943 \qquad s = 0.342 \qquad F = 31.9$

where $\underline{n}$ is the number of compounds, $\underline{r}$ is the multiple correlation coefficient, $\underline{s}$ is the standard error of the estimate, and $\underline{F}$ is the Fisher-test value.

The mathematical statistical characteristics (F-test, $\underline{s}$ and $\underline{r}$ values) show that we could find a reliable relationship between the chromatographic retention data using acetonitrile as the organic modifier and the 1-octanol/water shake-flask partition data for both columns used. The ratio of the parameters

living systems, except some special cases (e.g. central nervous system processes) where other liquid-liquid partition systems serve as a better model /7/.

The traditional, time-consuming shake-flask method for measuring log $K_{o/w}$ values is gradually replaced by reversed-phase high-performance liquid chromatographic (RP-HPLC) methods /8-13/. During the RP-HPLC retention the hydrophobic character of the solute is the predominant factor and the logarithm of the capacity factor (log k') of the compounds is directly proportional to their hydrophobicity.

In a previous paper /14/ it was pointed out that the log k' values determined in a reversed-phase chromatographic system show good correlation with the log $K_{o/w}$ values when structurally related compounds are measured or when the chromatographic partition system in which the log k' values were determined is similar enough to the 1-octanol/water partition system.

If 1-octanol and water are used in chromatography as proposed by Mirrlees et al. /10/ and Unger et al. /11/, the chromatographic conditions cannot be adjusted to compounds having very high or very low log $K_{o/w}$ values. Moreover, it is difficult to achieve a constant coating of the original stationary phase by 1-octanol.

In this paper a method is presented for finding an optimum reversed-phase chromatographic system (without 1-octanol) in which the log k' values determined for structurally unrelated compounds show the closest correlation with the log $K_{o/w}$ values; i.e., the differences between the measured log $K_{o/w}$ values and the log $K_{o/w}$ values estimated from the log k' values are minimal.

The same method can be applied for finding a reversed-phase chromatographic system by which optional partition systems (even a biological one) can be best modelled.

THEORETICAL APPROACH

Collander/15/ described the relationship between the partition coefficients measured in two different partition systems:

$$\log K_1 = a_1 \log K_2 + a_2 \quad (1)$$

where K refers to the partition coefficients, subscript 1 and 2 refer to two different liquid-liquid partition systems and a_1 and a_2 are constants characteristic of the systems used. Leo /16/ pointed out that eq. 1 was valid only if either the compounds or the two partition systems were similar in character.

When $\log K_2$ values of different solutes refer to a chromatographic partition system it is known that the $\log K_2$ values are proportional to the log k' values of the compounds according to eq. 2:

$$\log K_2 = \log k' - \log (V_s/V_m) \quad (2)$$

where V_s/V_m is the ratio of the stationary and the mobile phase volumes, which are constant in a given chromatographic system. If the log k' values are introduced into eq. 1, eq. 3 can be obtained:

$$\log K_1 = a_1 \log k' - a_1 \log (V_s/V_m) + a_2 \quad (3)$$

It should be mentioned that different log k' values can be obtained for the same compound when different eluent mixtures are used. It has also been described previously /17/ that the log k' values of compounds depend on the organic phase concentration of the eluent according to eq. 4:

$$\log k' = \underline{m}\ OP\% + \log k'_o \quad (4)$$

where OP% is the organic phase concentration of the eluent, $\underline{m}$ and $\log k'_o$ are constant for a given compound and organic phase. From the measured log k' values at different OP% values (for a given compound) log k' values can be calculated for any organic phase concentration.

If we combine eqs 4, 2 and 1, eq. 5 can be derived:

$$\log K_1 = a_1\ OP\%\ \underline{m} + a_1 \log k'_o - a_1 \log (V_s/V_m) + a_2 \quad (5)$$

The values of $\underline{m}$ and log k'_o can be calculated from the measured log k' *vs.* OP% data, while log K_1 values determined by shake-flask method are available for a large number of compounds. By introducing the data of log K_1, $\underline{m}$ and log k'_o for various compounds in eq. 5 the parameters a_1 OP%, a_1 and (a_1 log $(V_s/V_m)+a_2$) can be optimized by the least squares method. If the optimized a_1 OP% and a_1 are known, the value of the optimum OP%, denoted as $OP\%_x$, can be calculated. The corresponding log k' (log k'_x) can be expressed from eq. 4.

If we know $OP\%_x$, the log k'_x value of any other compound can be measured with this optimum eluent composition. These log k'_x values will show the closest correlation with the log K_1 values.

In some cases the log k'_x values of a compound cannot be measured at the optimum eluent composition ($OP\%_x$) because of very high or very low retention times. Then log k' values can be measured by using more convenient eluent compositions and the $\underline{m}$ and log k'_o values calculated from eq. 4 may be applied for the estimation of log k'_x.

MATERIALS AND METHODS

Apparatus

The RP-HPLC system consisted od a Liquopump Model 312 pump (Labor MIM, Budapest, Hungary) with modified electronics and Altex (Cotati, Calif., USA) check valves, a Rheodyne (Cotati, Calif., USA) Model 7010 sample injection valve with 20 µL loop, a 250 x 4.6 mm column with LiChrosorb RP-18 (dp 10 µm) (Chromatronix Inc., Calif., USA) stationary phase and a 50 x 4.6 mm column with Supelcosil LC-18 (dp 5 µm) (Supelco, Bellefonte, Penn., USA) stationary phase, and an ISCO (Lincoln, Nebraska, USA) Model 226 absorbance monitor with 254 nm source screen and an ENDIM (Berlin, GDR) Model 621.01 recorder. Retention time measurements, data processing and mathematical statistical calculations were carried out on an Apple II microcomputer (Apple Computer Inc., Cupertino, Calif., USA).

Materials

The model compounds were the following: sulfadimidine (Alkaloida Pharmaceutical Works, Tiszavasvár, Hungary), nicotinamide and acetazolamide (Serva, Heidelberg, FRG), sulfamethoxypyridazine and methylsalicylate (Sigma, St. Louis, Mo., USA), caffeine (Fluka AG, Buchs, Schweiz), chloramphenicol (EGYT Pharmaceutical Works, Budapest, Hungary), salicylamide and phenacetine (Bayer, Leverkusen, FRG), acetanilide (Merck, Darmstadt, FRG), and benzaldehyde (Carlo Erba, Milano, Italy). The samples gave single peaks on the chromatograms. Their log $K_{o/w}$ values taken from the compilation of Hansch and Leo /18/ are listed in Table 1. Acetonitrile and other chemicals (reagent grade) were purchased from REANAL Fine Chemical Works (Budapest, Hungary). Acetonitrile was further purified for HPLC purposes.

Operational conditions of the HPLC measurements

The eluents used were various (v/v) mixtures of acetonitrile and 0.05 M KH_2PO_4. The acetonitrile concentration of the eluent varied from 5 to 75% by 5% steps. The concentration was always adjusted to give an appropriate retention time of the given compounds, i.e. the log k' values ranged from -0.5 to 0.5. The eluent contained 5 to 20% acetonitrile when nicotinamide and acetazolamide, 10 to 25% when caffeine, 15 to 30% when sulfadimidine, 20 to 35% when sulfamethoxypyridazine, 25 to 40% when salicylamide, 30 to 45% when chloramphenicol and acetanilide, 35 to 50% when phenacetine, 45 to 60% when benzaldehyde and 60 to 75% when methylsalicylate were measured. The eluent mixtures were prepared by pouring the acetonitrile into a graduated cylinder and the final volume of 500 mL was adjusted with 0.05M KH_2PO_4.

Degassing was carried out under vacuum while stirring with an electric mixer (Superstir, Labor MIM, Hungary). The flow rate was 0.50 mL/min and 1.50 mL/min with Supelcosil LC-18 and LiChrosorb RP-18 columns, respectively, Sample: 1 mg of the

compounds tested and 2 mg $NaNO_3$ were dissolved in 5 mL eluent and 20 µL of the solution was injected into the column.

Calculation of data

The retention time of $NaNO_3$ was regarded as the dead time (t_o). Both retention time (t_R) and the dead time were measured three times. The average log k' values ($\log/(t_R-t_o)/t_o/$) were calculated. Log k' values obtained with different eluent compositions were correlated with the acetonitrile concentration of the eluent according to eq. 4 and the $\underline{m}$ and log k'_o values were calculated for each compound. Then the log $K_{o/w}$ values and the $\underline{m}$ and log k'_o values were introduced into eq. 5. The parameters a_1 OP%, a_1 and (a_1 log (V_s/V_m) + a_2) were calculated by linear regression analysis.

RESULTS AND DISCUSSION

The reversed-phase liquid chromatographic retention data of eleven compounds are listed in Table 1.

The correlation coefficients of log k' $\underline{vs}$. OP% values (cf. eq. 4) were higher than 0.98 for each compound. Therefore, the $\underline{m}$ and log k'_o values referring to the two reversed-phase columns, listed in Table 1, can be properly calculated.

The values of Table 1 were introduced into eq. 5. The linear regression analysis gave the optimum values for the parameters of eq. 5. Thus, we can write eq. 5 for the two columns as follows:

Supelcosil LC-18 column:

$$\log K_{o/w} = 27.34\ \underline{m} + 0.815 \log k'_o + 1.166 \qquad (6)$$

$n = 11 \qquad r = 0.949 \qquad s = 0.324 \qquad F = 36.1$

of m and log k'_o in eqs 6 and 7 gives the optimum organic phase concentration according to the considerations described after eq. 5. The optimum eluent composition was found to be 33.54% (v/v) and 30.14% (v/v) acetonitrile for Supelcosil LC-18 and LiChrosorb RP-18 stationary phases, respectively.

The log k' values measured with about 30% acetonitrile in the eluent (log k'_x) should have the closest correlation with the log $K_{o/w}$ values and this is what we found (cf. Fig. 1.). It is worthwhile mentioning that 30% acetonitrile as an eluent served as a good model for characterizing the 1-octanol/water partition, irrespective of the differences in the length and type of the two columns.

The mathematical-statistical characteristics of the log $K_{o/w}$ vs. log k' relationship (eq. 3) were calculated for a series of eluent mixtures ranging from 10 to 90% acetonitrile concentration. Calculations were performed for all eleven compounds tested. In Fig. 1. only the plot of the s values vs. OP% is presented. The smallest residual error was obtained at the organic phase concentrations found as optimum. The significant increase of the s value at other OP% values show that any other eluent composition is a poorer model of the 1-octanol/water partition system.

It is worthwhile mentioning that the values of the residual error of the log k' vs. log $K_{o/w}$ relationship were as high as 0.764 on Supelcosil LC-18 and 0.533 on LiChrosorb RP-18 stationary phases when the log k' values extrapolated to 0% acetonitrile concentration, i.e. log k'_o, were considered. This suggests that the log k'_o values as proposed by Harnisch et al. /17/ for the estimation of the log $K_{o/w}$ values cannot be used when structurally unrelated compounds are tested.

It should be pointed out that the above-described optimization method can be applied to find the optimum chromatographic partition system for modelling any other partition systems even if structurally unrelated compounds are investigated.

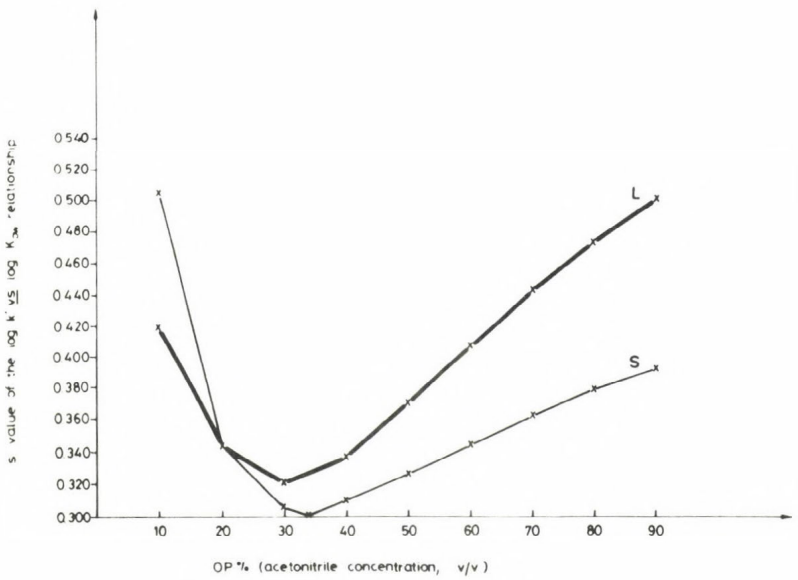

Fig. 1. Plot of s values for the log $K_{O/W}$ vs. log k' relationship as a function of acetonitrile concentration in the eluent*

*For OP% values used of the individual compounds cf. Methods. (S denotes Supelcosil LC-18 and L denotes LiChrosorb RP-18 stationary phases).

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The Apple II microcomputer was kindly provided by Dimes Group Inc., California, USA. The injection valve, the absorbance monitor and the LiChrosorb RP-18 column were kindly supplied by Chromatronix Inc., California, USA. The Supelcosil LC-18 column was a gift of Supelco Inc., USA.

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SILICONE RUBBER WALL-JET ELECTRODE IN HYDRODYNAMIC VOLTAMMETRY
(Comparison of various carbon electrodes)

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SUMMARY

Carbon-paste-, graphite embedded in silicone rubber- and glassy carbon electrodes are compared on experimental basis. It was proven that silicone rubber impregnated graphite (SR-graphite) electrodes display a residual current identical to that of glassy carbon, however, sensitivity is several times larger than that of glassy carbon electrodes. Examples are shown to prove the use of the SR-graphite electrodes as detectors for flow-injection system. The results also prove that the technique can be used to measure compounds in a certain very low concentration range with good accuracy the oxidation product of which in larger concentrations forms insulating films on the electrode surface.

INTRODUCTION

Different graphite-based electrodes were first employed for voltametric detectors at the end of the fifties, and, due to the fundamental work of Adams and co-workers as well as Farsang and co-workers /1-5/, they have gradually been replacing metal electrodes in voltametric analysis. It has been regarded as an advantage of these electrodes that the physical or chemical deactivation of the electrode surface could easily be overcome by renewing the electrode surface by mechanical or electrochemical treatment.

Carbon-paste electrodes were considered to be superior to the other types of carbon electrodes in having lower residual current and noise, while their drawback is the difficulties to make their surface sufficiently planar and smooth. Furthermore, carbon-paste electrodes may suffer from mechanical instability.

Recently Wang and co-workers reported on the modification of carbon-paste electrode with cation-exchanger resin with the aim of increasing the sensitivity in differential pulse voltammetry /6/.

Pungor and co-workers /7-9/ have introduced silicone rubber incorporated graphite electrodes (SR-graphite electrodes) for voltammetry. The main advantage of this new electrode was its mechanical stability, although it had a comparatively large residual current as a drawback.

In the meanwhile the glassy carbon /10-11/ has started to spread opening up new perspectives in analytical chemistry. However, it remained to be a question whether glassy carbon could be replaced by SR-graphite electrodes in aqueous volta - metric and amperometric measurements. Recently Štulik and co-workers /12, 13/ reported on carbon paste for voltametric detectors in high-performance liquid chromatography and found carbon pastes superior in many respects to other carbon electrodes. However, among carbone-paste electrodes with solid matrix they have found SR-impregnated graphite quite poor in performance characteristics. This report contradicted our earlier experiences, so we took up the matter once again. This paper aims to report on the comperative study of glassy carbon, carbon paste treated with silicone oil and graphite embedded in silicone rubber (SR-graphite) based electrodes as voltametric detectors in flow-injection analysis.

PRACTICAL PART

Measuring techniques

Flow-injecting system (Fig. 1) was used to test the different carbon electrodes. The construction of flow-injection system in principle is identical with that of a liquid chromatograph with the exception of a column.

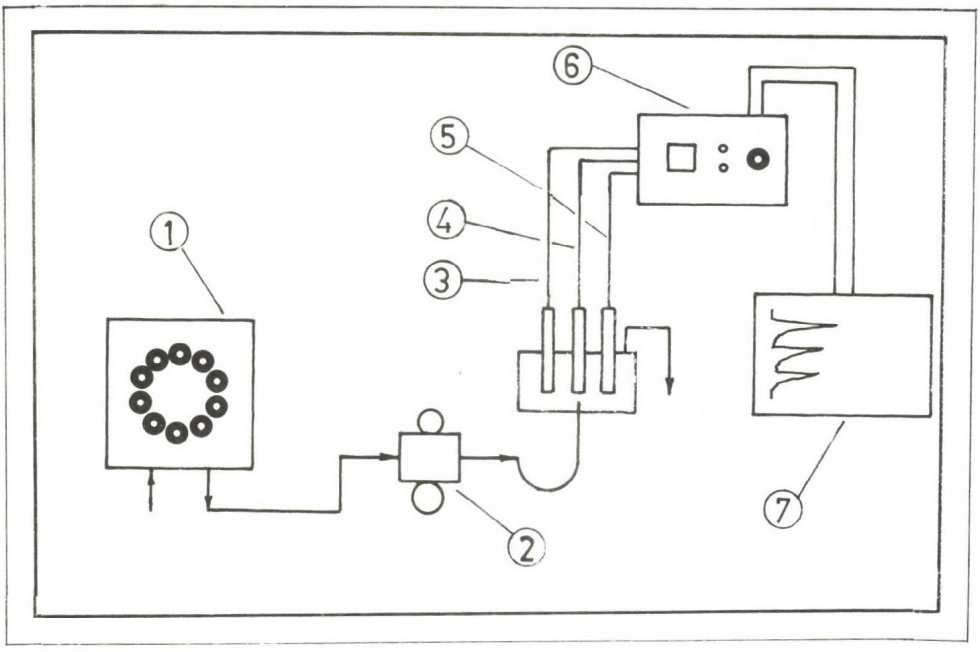

Fig. 1. Schematic diagram of a flow injection device
1. Peristaltic pump
2. Injector
3. Auxiliary electrode
4. Working electrode
5. Reference electrode
6. Potentiostat

Dopamine and Serotonine were used as model substances while the basic electrolyte was Na acetate - citric acid buffer with pH = 5.2, into which 1 mM Na_2EDTA was added.

Apparatus

Measuring cell incorporating the wall-jet working electrode is shown in Fig. 2 /14/. A constant voltage was supplied with the help of a home-made potentiostat-amplifier. The flow of the background electrolyte was ensured by an LKB Multiperpex 2115 peristaltic pump, while the sample was introduced with a Pierce MTS.S.V. 24144 type injector with double loops. The volumes injected were either 40 or 120 µl.

Recordings were made with an Omni Scribe recorder. SEM investigations were done with a JEOL JSM 50/A type instrument.

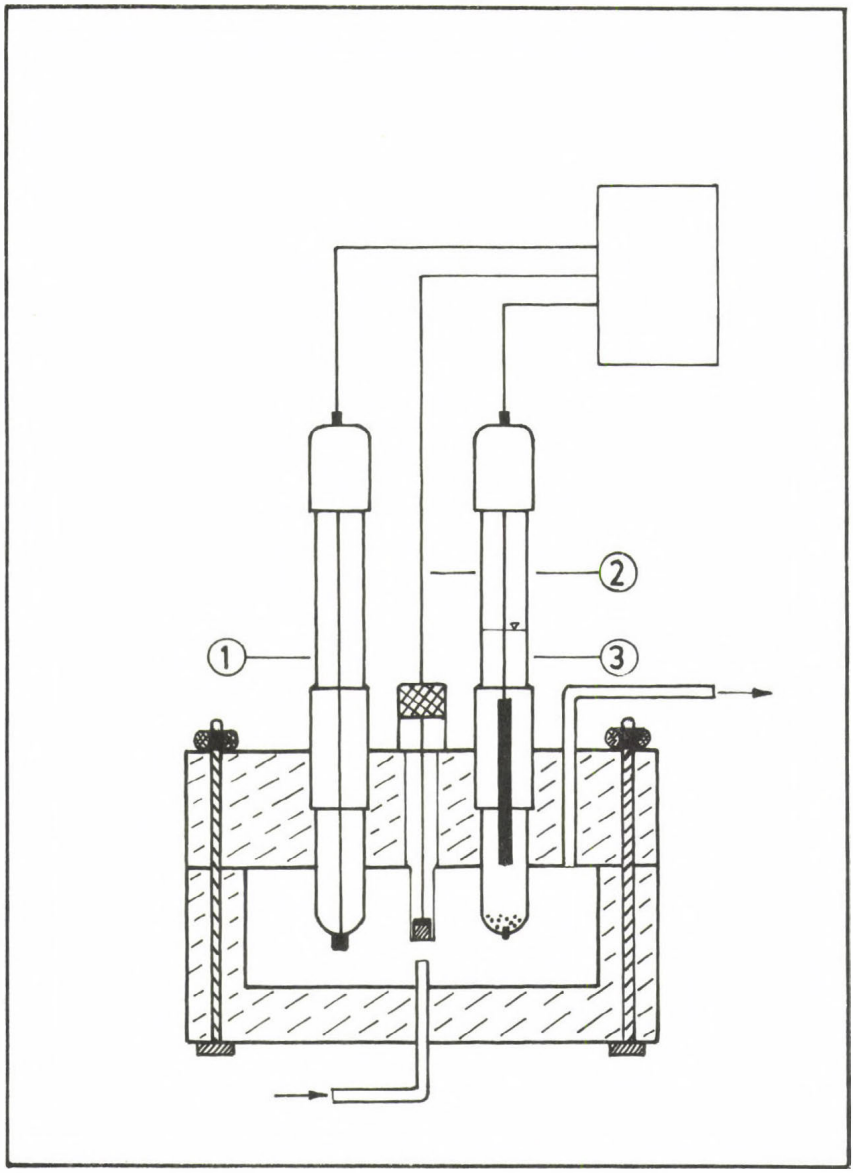

Fig. 2. Wall-jet voltametric detector cell
1. Auxiliary electrode
2. Working electrode
3. Reference electrode

Accelerating voltage was 20 kV. The investigated surfaces were treated with Au vapour.

Preparation of the electrode

Carbon powder with a particle size of less than 50 μm was mixed with polysiloxane (K: 1800) and with a cross-linking agent in order to prepare silicone rubber membrane sheets.

The working electrodes were prepared with the use of membranes with 4 mm diameter, a silicone rubber glue and appropriate electric contact was cared for.

SR electrodes were also prepared according to the procedure described by Štulik /12/ and similar poor results were obtained.

RESULTS AND DISCUSSION

Electrochemical investigations were made with three types of carbon-based electrodes. During our investigations the geometric surface of the electrodes were kept constant and, under identical conditions, the electrode sensitivity was determined and residual currents were noted. Table 1 presents the results. The results show that silicone rubber based graphite electrode has residual currents similar to those of glassy carbon, while its sensitivity is several times greater than that of the glassy carbon. Table 1 also shows that glassy carbon displays higher sensitivity than carbon paste electrode, while the residual current is smaller than in the case of carbon paste.

Table 1.

Electrode material	Residual current nA	Resistance Ω	Sensitivity nA/ng	Physical surface mm^2
Glassy carbon	~1	2	~3	19.6
Carbon paste	~3	5	~2	12.6
SR-graphite	~1	4	~10	12.6

Assuming that the surface of the carbon paste electrode is proportional to the percentage of carbon used for the preparation, the difference between the apparent and actual surfaces suggest that its sensitivity is almost identical to that of glassy carbon.

This leads to the following question: could the sensitivity increase of the SR graphite electrode be explained by the significantly larger actual surface area compared to the apparent surface? To answer this question electron micrographs were taken on the adequately prepared surfaces of glassy carbon and silicon- rubber membranes.

Figs 3 and 4 display the electron micrographs in identical magnification. It can be seen that in this magnification glassy carbon has a completely smooth surface, while in the case of SR graphite the surface is not smooth and 5-10 μm particles are protruding from the surface.

The performance characteristics of SR graphite electrodes have been evaluated with the flow-injecting technique. Investi-

Fig. 3. Electron micrograph of a SR-graphite electrode surface
Magnification: 1800

gations were limited to two test materials. Oxidation product of one did not form an insulating film on the electrode (Dopamine) while that of the other (Serotonine) is known to create an insulating film during electrochemical oxidation process.

Investigations with Dopamine covered the range between pg and a few ng. As presented in Figure 5 the peak-shaped signals obtained are well reproducible. Figure 6 proves that a linear function exists between the amount of the substance injected and the peak height of signals within several orders of magnitude if equal volumes are injected. Otherwise, only the area under the peak is proportional to the amount injected due to the different residence time.

The investigations with Serotonine were to serve two purposes. One was to find out whether when using the injecting technique there exist a concentration range where oxidation film does not appear. The other was to find out how the coating film can be released from the electrode surface.

Figure 7 shows that according to our investigations no insulating film is formed on the electrode surface on the

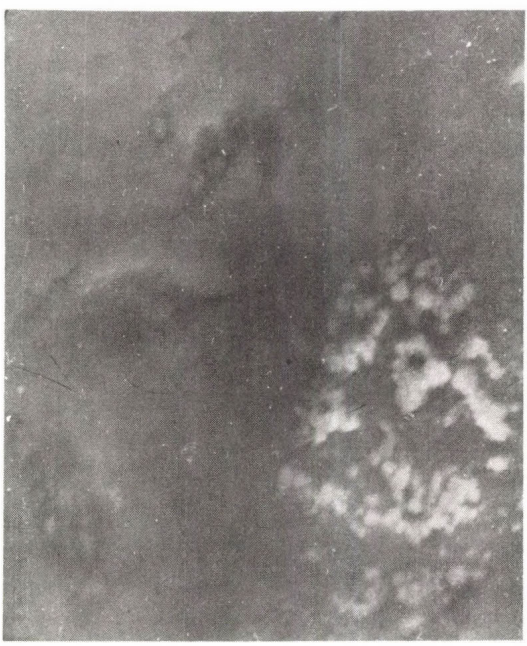

Fig. 4. Electron micrograph of a glassy carbon electrode surface
Magnification: 1800

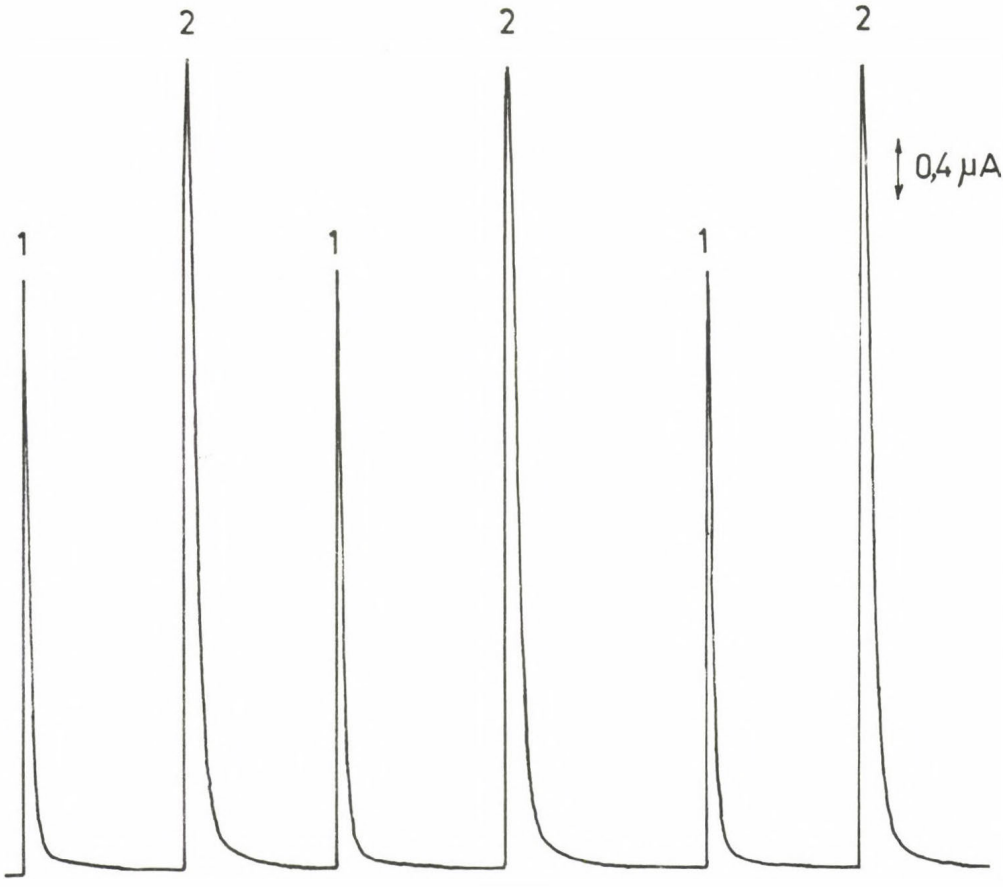

Fig. 5. Recorder trace obtained with a voltametric detector on injecting different amounts of Dopamine into a carrier stream
U_{pol}: + 0.65 V (vs.SCE) Working electrode: SR-graphite
Sensitivity: 10 µA/V Reference electrode: SCE
Flow-rate: 1 ml/min Auxilary electrode: Pt
Amount injected: 1. 600 ng Dopamine
2. 1.8 µg Dopamine

border between the pg and ng ranges. This can be explained by the low concentration of the electroactive-substance and the flowcondition existing in the wall-jet cell. In the meanwhile Figure 8 proves that films are formed on the electrode surface in larger concentration ranges, deactivating the electrode surface and reducing the electrode sensitivity.

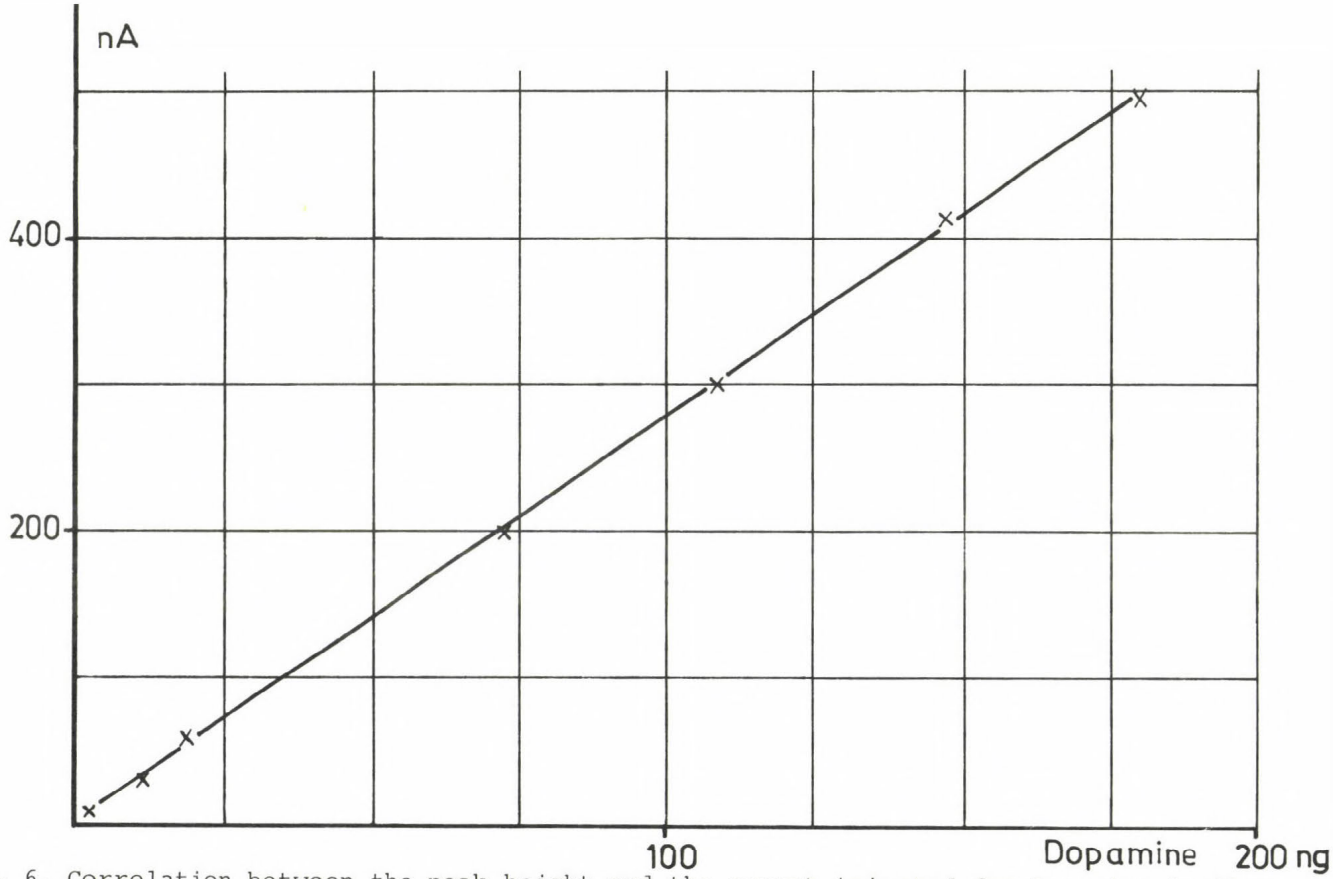

Fig. 6. Correlation between the peak height and the amount injected for Dopamine in flow injection analysis using voltametric detection
Working electrode: SR-graphite
Auxiliary electrode: Pt
Reference electrode: SCE
U_{pol} = + 0.65 V (vs. SCE)

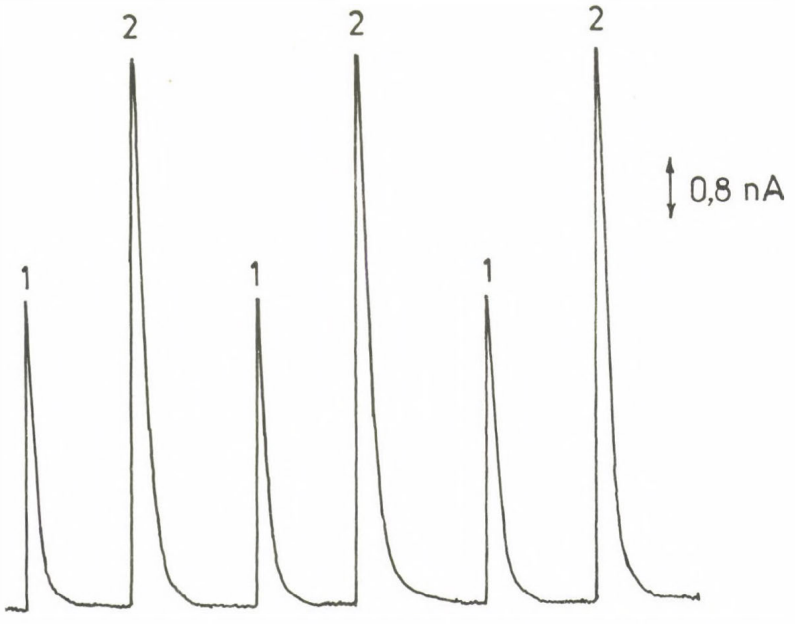

Fig. 7. Reproducibility of the recorder signals obtained on injecting Serotonine in low concentrations
U_{pol}: + 0.65 V (s.v SCE) Amount injected: 1. 700 pg
Sensitivity: 20 nA/V 2. 2.1 ng
Flow rate: 1 ml/min

Based on our earlier experiences we attempted to inject organic solvent into the system to make free the electrode surface from the insulating film. In harmony with former experiences acetone served this purpose. After injecting acetone on the film we investigated the electrode response with Dopamine which is not a film-forming compound. Figure 9 displays the obtained effects proving an increase in sensitivity as a result of the partial dissolution of the deactivating film by acetone; as consequence of this, the Dopamine peaks starts to increase.

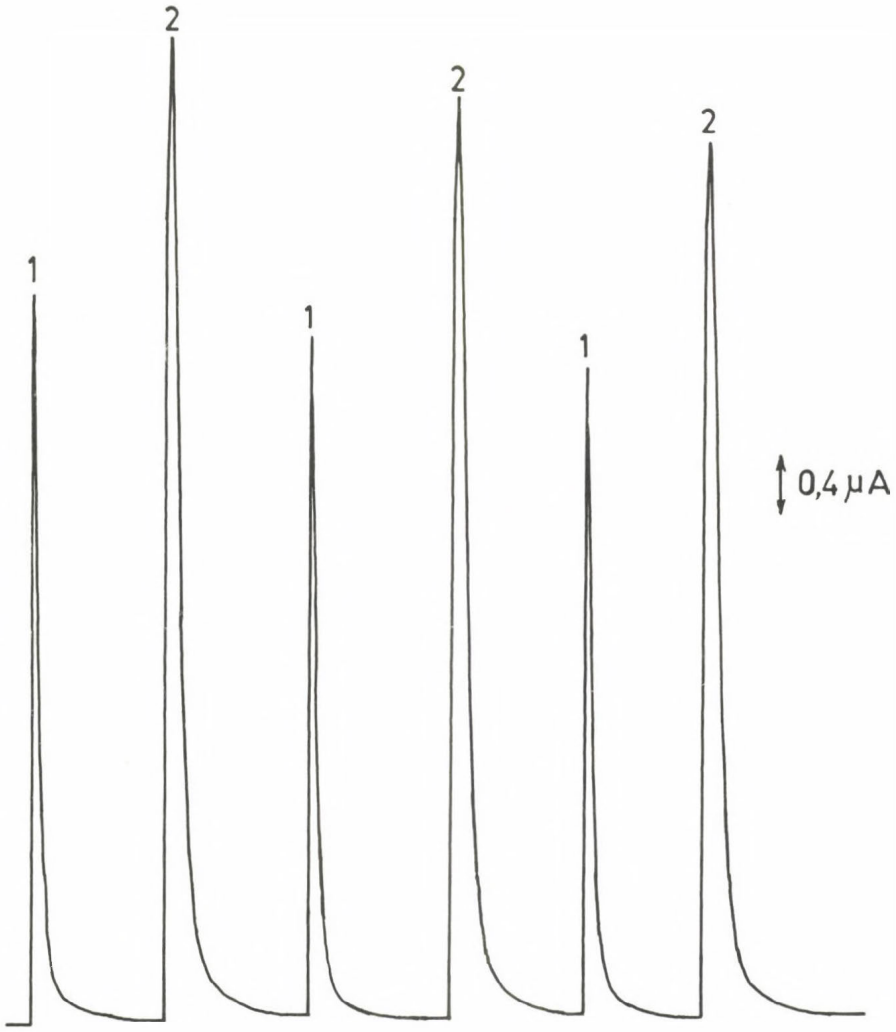

Fig. 8. Recorder trace indicating surface reactions between the electrode material and the Serotonine sample injected at relatively high concentrations
Working electrode: SR-graphite
Reference electrode: SCE
Auxiliary electrode: Pt
$U_{pol.}$: + 0.65 V Amount injected: 1. 1.62 µg
Sensitivity: 10 µA/V 2. 4.86 µg

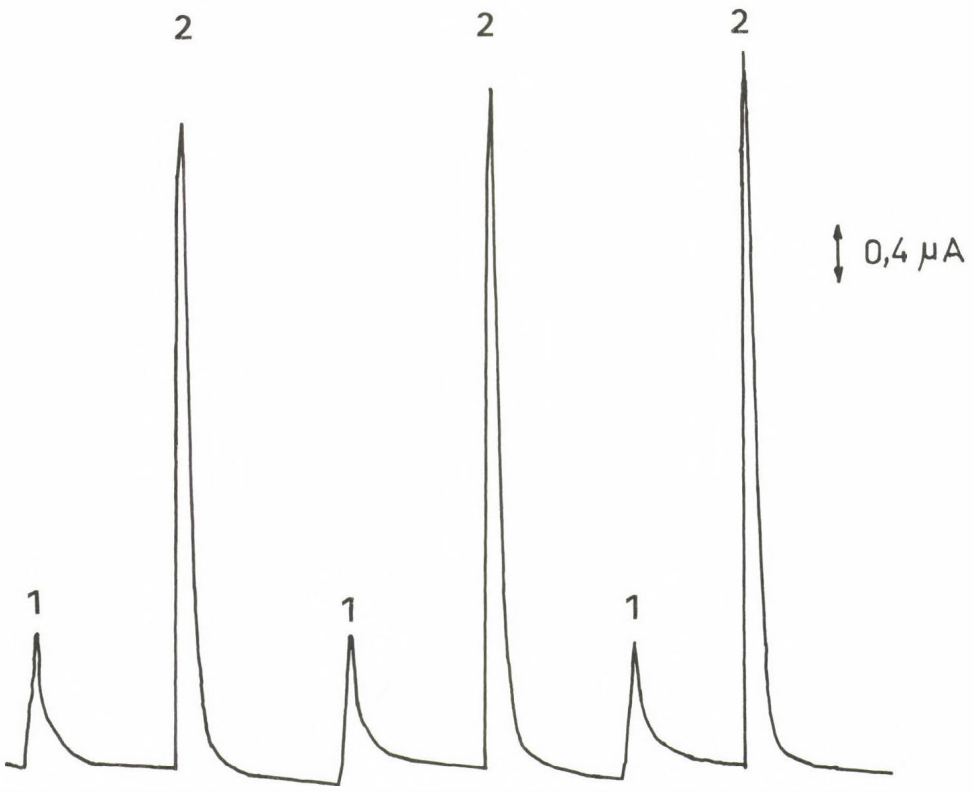

Fig. 9. The effect of acetone injection on the Dopamine signal recorded with partially deactivated SR-graphite electrode
$U_{pol.}$: + 0.65 V (v.s. SCE) Amount injected: 1.8 µg
Sensitivity: 10 µA/V
Flow-rate: 1 ml/min

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STATIONARY PHASES FOR CHROMATOGRAPHY AND THEIR INTERACTIONS

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H. Kalász and L.S. Ettre (Eds)

THE SYNTHESIS AND PERFORMANCE OF A CHENICALLY BONDED SULFUR HETEROCYCLIC STATIONARY PHASE FOR HPLC

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SUMMARY

A chemically bonded phase for reversed-phase liquid chromatography, based on a silane, substituted with propylthiophene was synthesised. The phase exhibits selective properties, demonstrated on aromatic isologues. A comparison is made between several reverse-phase materials.

INTRODUCTION

Research concerning chemically bonded phases for reversed-phase liquid chromatography has during the last decade led to the development of a number of silanes, used as efficient non-polar phases. The most frequently used separation system has been the ODS-phase, consisting of an octadecyl carbon chain attached to silica gel. This phase can be characterized either as polymeric, mostly using a trichlorosilane during the synthesis, or as monomeric, using a monochlorosilane. The fact that this type of phase is still commonly regarded as the superior phase for the separation of a wide range of chemical compounds, can probably not be explained solely by the lack of development of new phases. Optimal chain length and good application techniques (surface coverage etc.) probably contribute towards the advantageous properties of the ODS.

Partition chromatography is the primary separation mechanism of the ODS-phase used in the reversed-phase mode, and thus

theoretically resulting in most ODS-phases having similar selectivity characteristics. The fact that this is not entirely true has been demonstrated in a number of publications /1-3/, where fairly nonpolar polyaromatic hydrocarbons (PAHs) have been shown to exhibit different elution orders, depending on varying manufacturing characteristics of the ODS-phase. This change in the selectivity has not necessarily been followed by a change in performance, indicating that a slight degree of mixed chromatography (i.e., separation mechanisms) is not detrimental to the chromatographic performance. Consideration of a small degree of adsorption chromatography must be included when studying the separation process of chemically bonded phases on silica gel /4-7/.

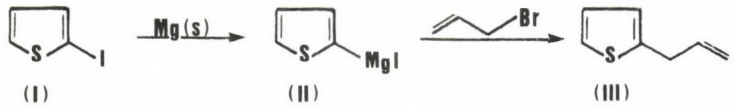
Achievement of selectivity is of special interest for the separation of compounds with slightly polar properties from non-polar compounds such as, for example, different kinds of aromatic isologues. Considering the fact that change in separation selectivity can be achieved, while maintaining the separation performance, this paper demonstrates the synthesis and utility of a slightly polar, chemically bonded phase for reversed-phase liquid chromatography. The phase is based on the introduction of an aromatic sulfur heteroatom by means of a thiophene ring attached to a propyl chain, chemically bonded to silica gel. It has recently been demonstrated that sulfur heterocyclic polyaromatic compounds (S-PAC) show regular retention behaviour with respect to their PAH isologues on reversed phase ODS-columns /8/. A complementary phase with reproducible slightly selective properties for reversed-phase HPLC is desirable for the analysis of S-PAC.

SYNTHESIS OF THE SILANE

A number of reaction paths were tested for the synthesis of thiopheno-propyl-trichloro-silane, the most successful of which is described here.

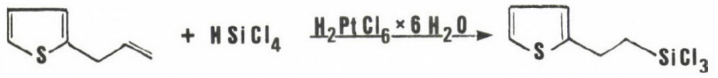
Initially, 2-iodo-thiophene was synthesised according to /9/. 2-Allylthiophene (III) was consequently synthesised by a

Grignard reaction utilizing 2-iodo-thiophene and allylbromide /10/.



(I) (II) (III)

The thiophene-propyl-trichlorosilane (V) was developed by a hydrosilylation reaction involving allylthiophene and trichlorosilane, using hexachloroplatinic acid as catalyst according to Mourey et al. /11/).



(IV) (V)

Final bonding to 10-µm silica gel was carried out by refluxing dry toluene containing the synthesised silane mixed with different percentages of propyl-trichlorosilane and pyridine /12/.

Consequently, the columns were packed using the slurry method.

APPARATUS

The columns were tested on a Schimadzu LC-4A equipped with a 8-µl variable UV-absorption detector (SPD-2AS). The detector response was A/D-converted and registered by an ABC-800 micro computer, performing all other signal treatment and plotting.

RESULTS

The two isologues fluorene and dibenzothiophene were chosen as test compounds in order to compare the selectivity of the thiophene phase with that of phases manufactured from pure

alkylsilanes. These two compounds are known to exhibit separation on different kinds of ODS-phases (Fig. 1), the dibenzothiophene eluting prior to the hydrocarbon. On increasing the polarity by introduction of a thiophene ring in the bonded phase, the selectivity was drastically changed. Thus, 10% thiophene phase and 90% propylsilane phase resulted in the separation of the test compounds with a different order of elution. This selectivity cannot fully be attributed to the chemically bonded thiophene ring, as a 100% propylsilane phase also caused a change in the order of elution (but with much poorer selectivity). Obviously, the distance from silanol groups on the silica gel to the solutes also plays an important role in this case, as mentioned in the introduction. This is a property which should be taken into consideration. Polar areas on the column material, probably unreacted silanol groups, play an important role in the polarity of a phase. This effect can be reduced or circumvented by the use of a high percentage of covering with additional end-capping.

Alternatively, the effect can be utilized for selectivity purposes by high reproducibility in the synthesis of phases. Nevertheless, a well-controlled introduction of a characteristic group, chemically bonded to an alkylsilane, can lead to desired and reproducible properties of the phase synthesised. The synthesis of a thiophene containing phase for separation in the reversed-phase mode has shown that specific separation properties can be achieved by the introduction of slightly polar, chemically bonded functional groups.

The capacity factors and the selectivity for fluorene and dibenzothiophene are shown in Table 1. The corresponding chromatograms, using the Vydac-ODS, propylthiophene-propyl (10/90) and the propylthiophene-propyl (50/50) are shown in figures 1-3. In the two latter cases, a mobile phase consisting of methanol-water (40/60) has been used, whereas in the separation on the Vydac-ODS, a mobile phase consisting of methanol-water (70/30) was used.

It is interesting to note that the introduction of only 10% propylthiophene in the propyl phase causes a drastic change in the chromatographic properties of the phase. The capacity

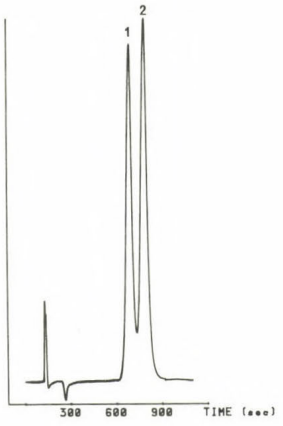


Fig. 1. Vydac ODS

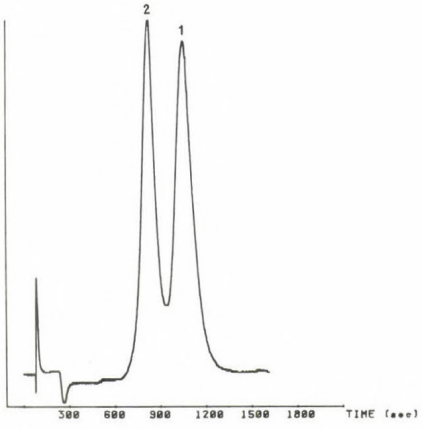
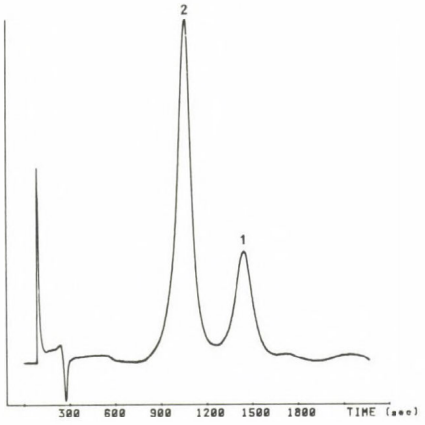


Fig. 2. Propylthiophene/ propyl 10/90



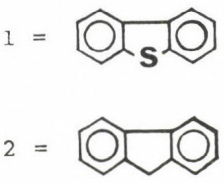


Fig. 3. Propylthiophene/propyl 50/50

Figures 1-3 exhibit the separation of dibenzothiophene (=1) and fluorene (=2). UV-absorption 254 nm. Flow rate 10 cm/min. (see table 1 for more details).

Table 1. Retention parameters for fluorene (Flu) and dibenzothiophene (DBT) on five columns

Col:	Vydac-ODS		Liq. ODS		Propyl		Thi./prop. (10/90)		Thi./prop. (50/50)	
	k'		k'		k'		k'		k'	
Flu	5.06	1.00	11.90	1.00	2.85	1.00	9.79	1.00	10.97	1.00
DBT	4.31	0.85	10.69	0.90	3.39	1.18	12.80	1.31	15.26	1.39
methanol water	70/30		70/30		40/60		40/60		40/60	

factors are increased three to four times and the selectivity is enhanced. Further increase in the ratio propylthiophene/propyl to 50/50 yields even better separation and increased k' values.

This result is not obvious. An increase in the capacity factor indicates an overall decrease in polarity of the phase. Thus, both the efficiency and the selectivity capability of the phase are increased after addition of only a few per cent propylthiophene-silane to a propyl-silane founded phase. This might be explained partly by the increase in chain length caused by the addition of the thiophene ring, giving an overall decrease in polarity coupled to the specific selectivity caused by the hetero atom.

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CYCLODEXTRIN POLYMERS AS STATIONARY PHASES IN THE LIQUID CHROMATOGRAPHY

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ABSTRACT

Cyclodextrins (CD's) can be coupled and crosslinked by di- or polyfunctional agents to form so-called cyclodextrin polymers (CDP's). A well-proved technical method has been developed for producing CDP's in form of small spherules (bead polymers). These products proved to be well utilizable in aqueous media as stationary phases, e.g. for the chromatographic separation of amino acids, alkaloids, moreover, also for the resolution of optical antipodes (enantiomer pairs). In these cases, the separation is mainly based upon the reversible formation of inclusion complexes.

WHAT ARE CYCLODEXTRINS AND CYCLODEXTRIN POLYMERS?

Cyclodextrins (CD's) are specific fermentation products (by Bacillus macerans enzymes) of starch. They are water-soluble, optical active (+), non-reducing cyclic oligosaccharides built up from six (α-CD), seven (β-CD) or eight (γ-CD) α-D-glucopyranose units.

Most popular is the β-cyclodextrin, produced in commercial scale by the CHINOIN Pharmaceutical and Chemical Works in Budapest, while α-CD and γ-CD are just pilot products.

The doughnut-shaped molecules of CD's bear all of their free primary OH groups on the one edge of the molecular ring, while the secondary free OH groups on the other edge of the molecular

ring. The external "surface" of these molecules is polar and hydrophil, while the internal cavity (approx. diameter is 5.7 Å, 7.8 Å and 9.5 Å for α-CD, β-CD and γ-CD, respectively) is apolar and slightly hydrophobic /1/.

Cyclodextrins can be coupled and crosslinked by appropriate di- or polyfunctional reagents to form so-called cyclodextrin polymers (CDP's). Most popular are CDP's of polyether-type, which can be produced by coupling CD's by diepoxides or epichlorhydrin (cf. Fig. 1).

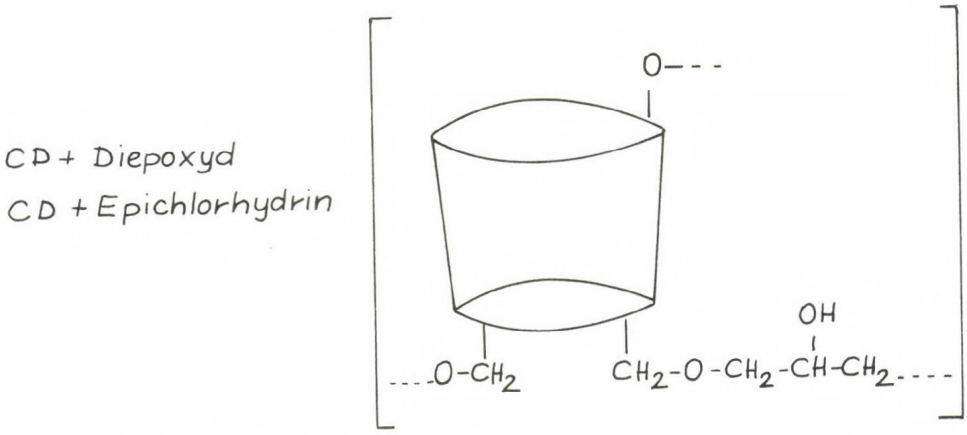

Fig. 1. Simplified structure scheme of a polyether-type CDP

For practical purposes, we have developed a technical process for the preparation of cross-linked cyclodextrin-polyvinylalcohol copolymers in form of small spherules (bead polymers) /2/. These polymer products are easy to handle, swell quickly in water, keep their form and elasticity in swollen state, too, are relatively stable against heating and microorganisms.

SEPARATIONS BY INCLUSION CHROMATOGRAPHY

Cyclodextrin polymers proved to be well utilizable as stationary phases in aqueous media for so-called inclusion

chromatographic separations /3/. The separation on CDP stationary phases is mainly based upon the ability of CD molecules ("host molecules"), to form inclusion complexes in aqueous media with molecules ("guest molecules") of appropriate size and geometry. The complexation is a reversible process (cf. Eq. 1) and can be quantitatively characterized by the dissociation constant K_d (cf. Eq. 2) or by its reciprocal, the so-called stability constant.

$$CD + S \rightleftharpoons CD \cdot S \quad , \qquad \text{(Eq. 1)}$$

$$K_d = \frac{[CD] \cdot [S]}{[CD \cdot S]} \quad , \qquad \text{(Eq. 2)}$$

where CD = cyclodextrin, S = substrate, CD·S = inclusion complex.

The retention of the substrate depends on the stability of the complex and alters with the polarity, hydrophobicity, size and geometry of the substrate molecule, the size of the internal cavity of the CD molecules, moreover, with the temperature and other experimental conditions (e.g. pH and the composition of the mobile phase).

It is to be mentioned that secondary effects, for example gel permeation and weak adsorption can also interfere with the complexation. In fortunate cases, these effects jointly increase the chromatographic separation.

Experimental conditions

Our chromatographic experiments were performed with the α-CDP, β-CDP and γ-CDP produced in bead form in our laboratory. Their main characteristics: CD-content 46-52%, PVA-content 0.3-0.4%, gel bed volume 4-5 ml/g CDP /4/.

The column chromatography was performed at atmospheric pressure using automatic equipment consisting of Pharmacia columns, LKB MultiPerpex pump, LKB UltroRac fraction collector, LKB Uvicord III absorptiometer and LKB flat-bed recorder. CDP was swollen in mildly acidic buffers, then filled and equilib-

rated in the columns. The same buffers were used for the dissolution of alkaloids and for the elution. Alkaloids and amino acids in the eluates were continuously detected by UV absorption.

Chromatography of amino acids /4/

As an example, Fig. 2 shows the complete separation of the five amino acids lysine, alanine, phenylalanine, tyrosine and tryptophan on a column packed with β-CDP.

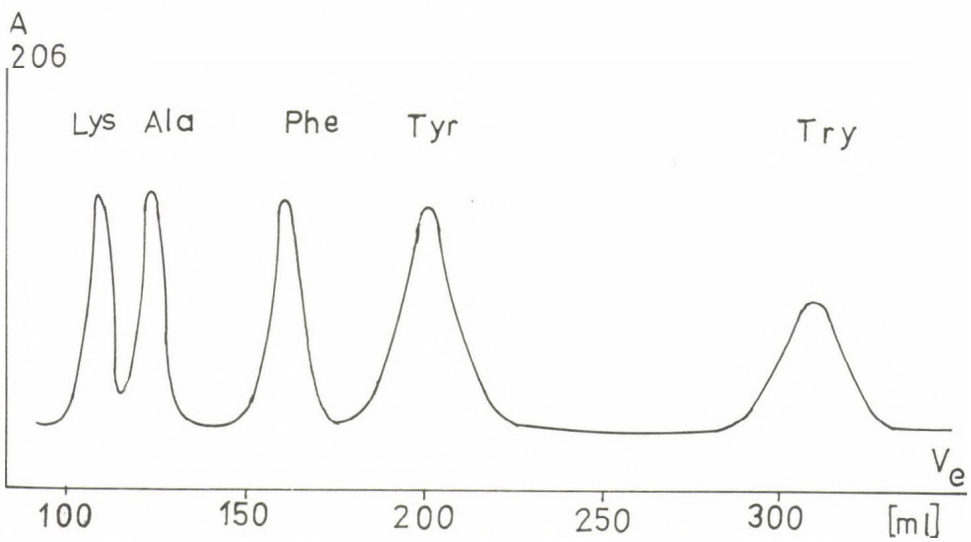

Fig. 2. Separation of α-amino acids on β-CDP: gel bed 1.6 x 88 cm, pH 5, phosphate buffer, flow rate 10 ml/h, 20°C

For characterisation of the efficiency, the height equivalents of a theoretical plate (HETP) are summarized for different flow velocities in Table 1.

A mixture of the former five amino acids was also chromatographed on α-CDP and γ-CDP stationary phases (cf. Fig. 3).

The chromatograms show that the best separation of these amino acids could be achieved on the column packed with β-CDP. On the other hand, tryptophan could be separated on α-CDP with the best selectivity.

Table 1

HETP values measured for α-amino acids on β-CDP at different flow rates (gel bed 1.6 x 88 cm, pH 5-6, phosphate buffer)

	HETP (mm)		
	10 ml/h	20 ml/h	40 ml/h
Tryptophan (0.2 mg)	0.7 - 0.8	0.7 - 0.8	1.7 - 1.9
Tyrosine (0.1 mg)	0.7 - 0.8	0.7 - 0.8	1.0 - 1.2
Phenylalanine (0.1 mg)	0.7 - 0.8	0.7 - 0.8	1.3 - 1.5
Alanine (2 mg)	0.4 - 0.5	0.4 - 0.5	0.7 - 0.8

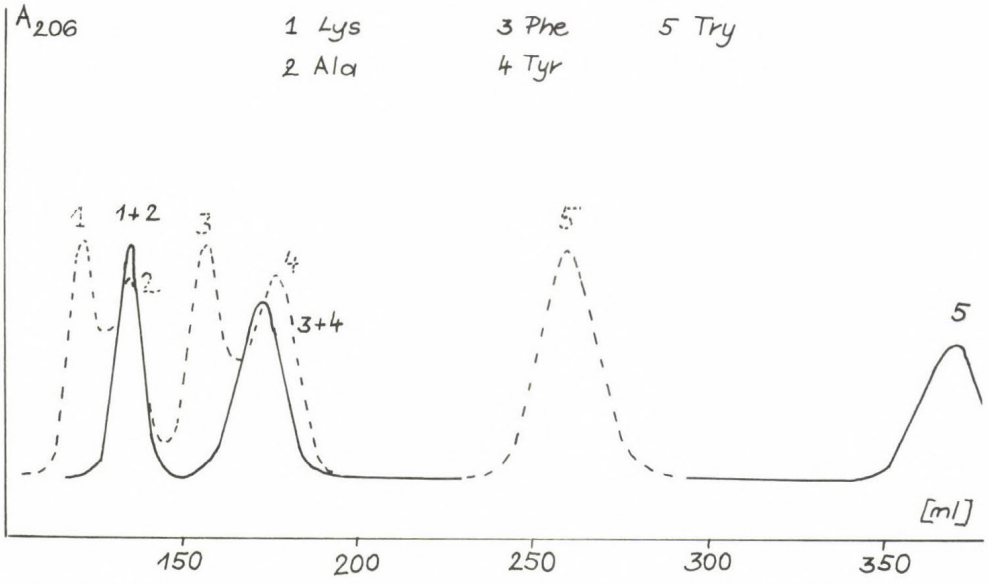

Fig. 3. Chromatography of α-amino acids on α-CDP (solid line) and on γ-CDP (dotted line): gel bed 1.6 x 88 cm, pH 5, phosphate buffer, flow rate 10 ml/h, room temperature

Also, further fifteen non-aromatic natural α-amino acids were chromatographed on β-CDP, but their peaks appeared all between or together with those of alanine and lysine.

Chromatography of alkaloids

Chromatographic behaviour of natural indole alkaloids was studied on CDP stationary phases. We found that many of them had different and unexpectedly high retention in mildly acidic buffer solutions at room temperature, which permitted their separation by inclusion chromatography /5/.

Fig. 4 shows the separation of two Vinca-alkaloids of very similar structure, (+)-vincamine and (+)-apovincamine.

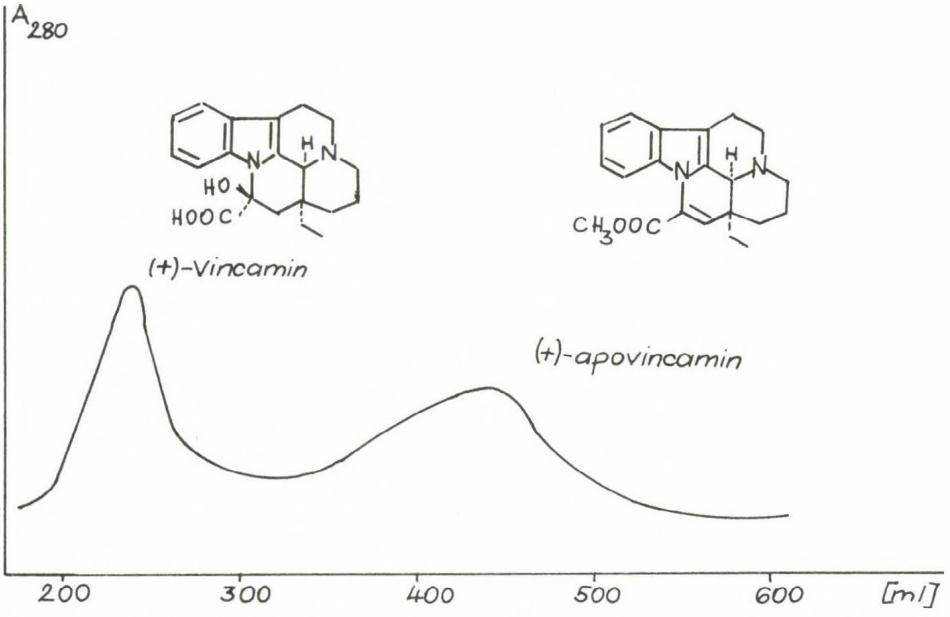

Fig. 4. Separation of (+)-vincamine (2 mg) and (+)-apovincamine (3 mg) on β-CDP: gel bed 1.6 x 90 cm, pH 5, citrate buffer, flow rate 80 ml/h, room temperature

Fig. 5 presents another example, the base-line separation of two Aspidosperma-alkaloids, (-)-aspidospermidine and (-)-vincadifformine.

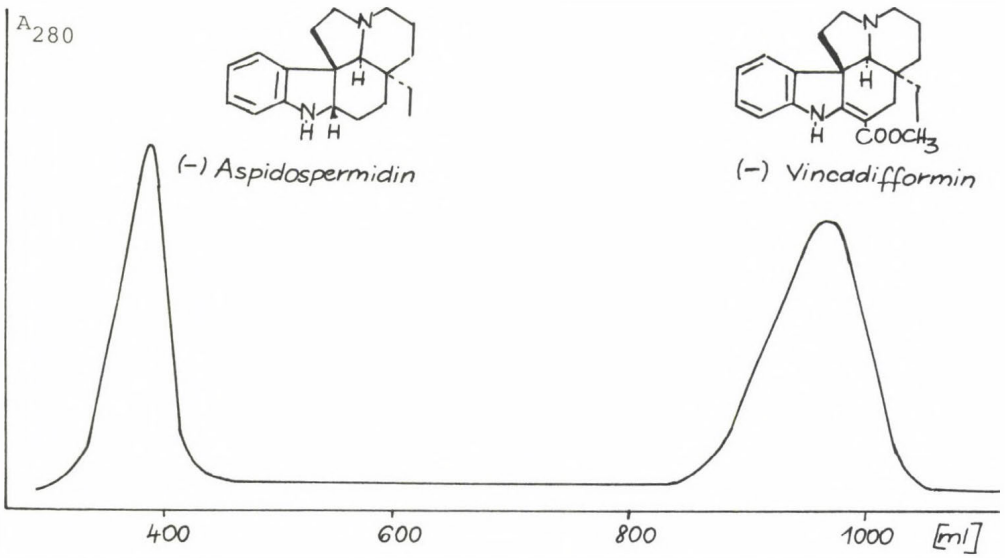

Fig. 5. Base-line separation of (-)-aspidospermidine (2 mg) and (-)-vincadifformine (4 mg) on β-CDP: gel bed 1.6 x 80 cm, 5.5 pH, phosphate buffer, room temperature

RESOLUTION OF RACEMATES BY INCLUSION CHROMATOGRAPHY

Under appropriate conditions, optical antipodes of chiralic compounds (i.e. enantiomers) can be separated on CDP stationary phases by inclusion chromatography. In this case, the separation can be attributed to the different stability of diastereomer inclusion complexes originated from the optical antipodes and the optical active (+)-cyclodextrin (cf. Eq. 3).

$$(+)\text{-CD} + (+)\text{-S} \rightleftharpoons (+)\text{-CD}.(+)\text{-S}$$

$$(+)\text{-CD} + (-)\text{-S} \rightleftharpoons (+)\text{-CD}\cdot(-)\text{-S} \qquad \text{(Eq. 3)}$$

Chances of chromatographic resolution by inclusion chromatography were systematically studied on a series of enantiomer pairs of indole alkaloids as model compounds and hopefully good results were achieved in both analytical and preparative scale /6/.

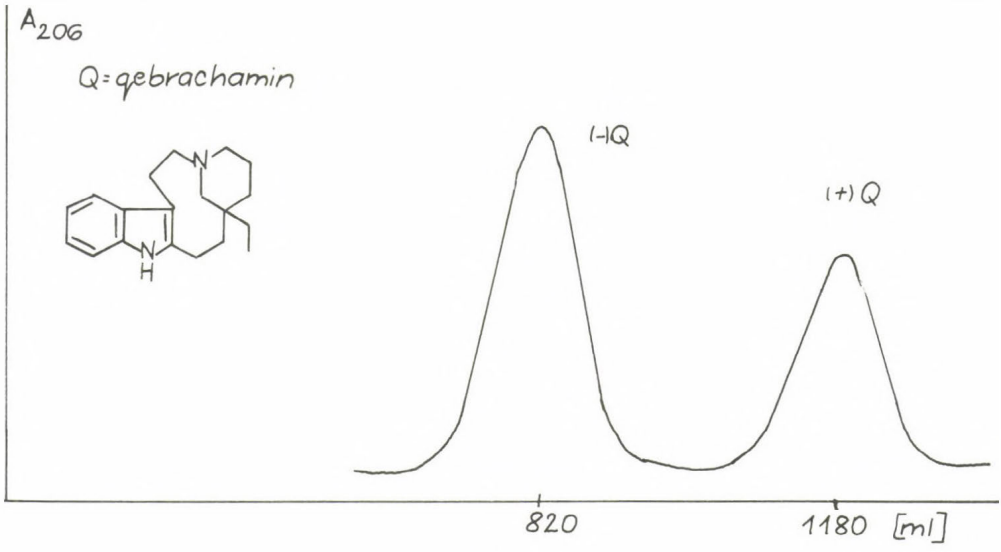

Fig. 6. Base-line resolution of (+)-quebrachamine (2 mg) and (-)-quebrachamine (2 mg) on β-CDP: gel bed 1.6 x 85 cm, pH 6.8, phosphate buffer, flow rate 50 ml/h, room temperature

Fig. 6 shows the analytical base-line separation of quebrachamine antipodes by inclusion chromatography on β-CDP.

For another example, the preparative chromatography of 500 mg racemic (±)-vincadifformine on β-CDP can be seen in Fig. 7.

The product isolated from the chromatographic fraction (A) was 230 mg crude (+)-vincadifformine, $[\alpha]_D^{20}$ = +510° (in ethanol); after recrystallisation from ethanol $[\alpha]_D^{20}$ = +580° (in ethanol). The optical purity was 98.3%. From the chromatographic fraction (B), 245 mg crude (-)-vincadifformine could be isolated, $[\alpha]_D^{20}$ = - 450° (in ethanol); after recrystallisation $[\alpha]_D^{20}$ = - 510° (in ethanol). The optical purity was 92.5%.

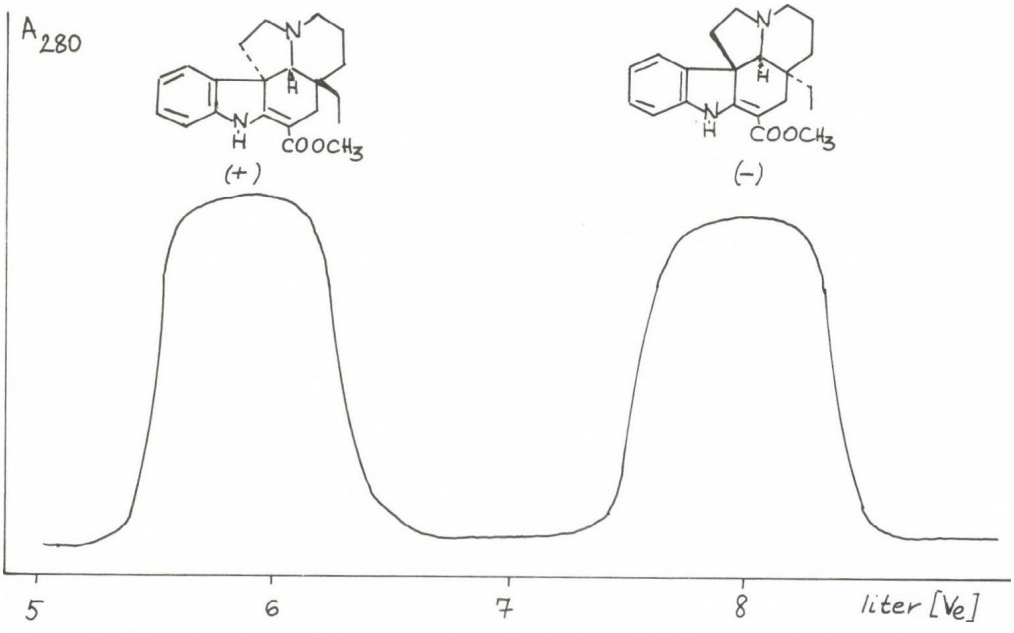

Fig. 7. Resolution of a racemic mixture of (+)-vincadifformine (250 mg) and (-)-vincadifformine (250 mg) in preparative scale on β-CDP: gel bed 5 x 90 cm, pH 5.5, phosphate buffer, Flow rate 300 ml/h, room temperature

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STERIC EFFECTS OF SUBSTITUENTS IN NORMAL-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The influence of the alkyl substituents of hydroxyaromatic, silicon-containing compounds on their retention on Silasorb 600, using n-hexane as the eluent, was investigated. These compounds were synthesized for the first time for these investigations. It is shown that the increase of the dimensions of the alkyl substituents results in a decrease of the retention of the compounds investigated. This is due to an increase in the steric hindrance of the oxygen atom in the dimethylalkoxysilyl substituent, significantly contributing to the retention. There is a correlation between the logarithms of the adjusted retention times of the investigated compounds and the steric constants of the alkyl groups in the alkoxy substituents.

INTRODUCTION

Silicon-containing phenols represent a relatively little investigated group of hydroxyaromatic compounds. Silicon-containing phenols have been utilized in the preparation of phenoplasts [1], stabilizers [2] or drugs [3,4].

The development of universal and efficient methods for the synthesis of such compounds requires a reliable analytical method. Chromatography is the most convenient method. The utilization of gas chromatography for this purpose is quite

often complicated by the thermal rearrangement of silicon-containing phenols [5]. The use of high-performance liquid chromatography (HPLC) makes it possible to avoid such difficulties and to carry out the control of the synthesis steps and the determination of the purity of the products at room temperature.

We have investigated the influence of the characteristics of silicon-containing compounds on their retention in HPLC. Three compound series, containing alkoxydimethylsilyl groups, were selected as the objects of the study.

EXPERIMENTAL

All the investigations were carried out on a Tsvet-304 liquid chromatograph equipped with a UV detector (254 nm) and stainless-steel columns (190 x 4 mm I.D.). Silasorb 600 (particle diameter: ca. 5 μm) (Lachema, Brno, Czechoslovakia) with a specific surface area of 600 m^2/g was used as the adsorbent. n-Hexane served as the eluent at a flow rate of 1 cm^3/min. All the measurements were carried out at room temperature (22°C). The samples (1 μl) were dilute solutions of the investigated compounds in n-hexane and were injected with a 10-μl syringe.

Details on the synthesis of the investigated silicon-containing compounds will be published separately.

RESULTS AND DISCUSSION

Table 1 gives the experimentally determined retention times (t_R) and the steric constants of Palm [$E^o_{s(Alk)}$ and $E^o_{s(CH_2Alk)}$] and Charton (v) [6] of the alkyl substituents for 12 silicon-containing compounds of the following three series:

I - aryloxysilanes (compounds 1-4),
II - silicon-containing phenols (compounds 5-8),
III - substituted disiloxydiphenyls (compounds 9-12).

As can be seen from Table 1, a general regularity exists for all three types of organosilicon compounds: the retention

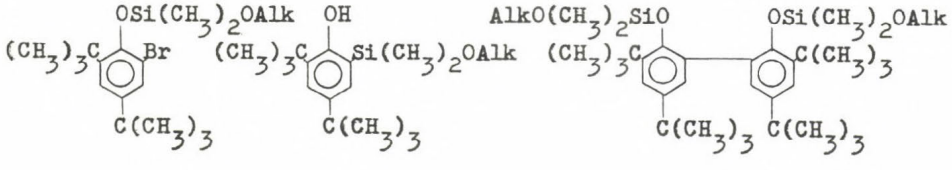
Table 1

Retention times (t_R) of silicon-containing compounds and Palm [$E^o_{s(Alk)}$ and $E^o_{s(CH_2Alk)}$] and Charton (v) steric constants of their alkyl substituents*.

Compound type**	No.	Alk group	t_R min	$E^o_{s(Alk)}$	$E^o_{s(CH_2Alk)}$	v
I	1	Methyl	13.95	0.00	-0.27	0.52
	2	Ethyl	9.83	-0.37	-0.56	0.56
	3	Isopropyl	6.35	-0.85	-1.13	0.76
	4	Tert.butyl	3.53	-2.14	-1.94	1.24
II	5	Methyl	11.83	0.00	-0.27	0.52
	6	Ethyl	9.32	-0.37	-0.56	0.56
	7	Isopropyl	7.27	-0.85	-1.13	0.76
	8	Tert.butyl	6.10	-2.14	-1.94	1.24
III	9	Methyl	212	0.00	-0.27	0.52
	10	Ethyl	94	-0.37	-0.56	0.56
	11	Isopropyl	40	-0.85	-1.13	0.76
	12	Tert.butyl	8.07	-2.14	-1.94	1.24

*Retention times were measured on Silasorb 600 using n-hexane as the mobile phase. The steric constants are taken from ref. [6].

**The structures of the compounds are:



time decreases when increasing the dimensions of the alkyl substituents. This regularity is graphically illustrated in Fig. 1 plotting the logarithm of the adjusted retention times (log t_R') against the number of carbon atoms (n_C) in the alkyl substituent of the investigated silicon-containing compounds.

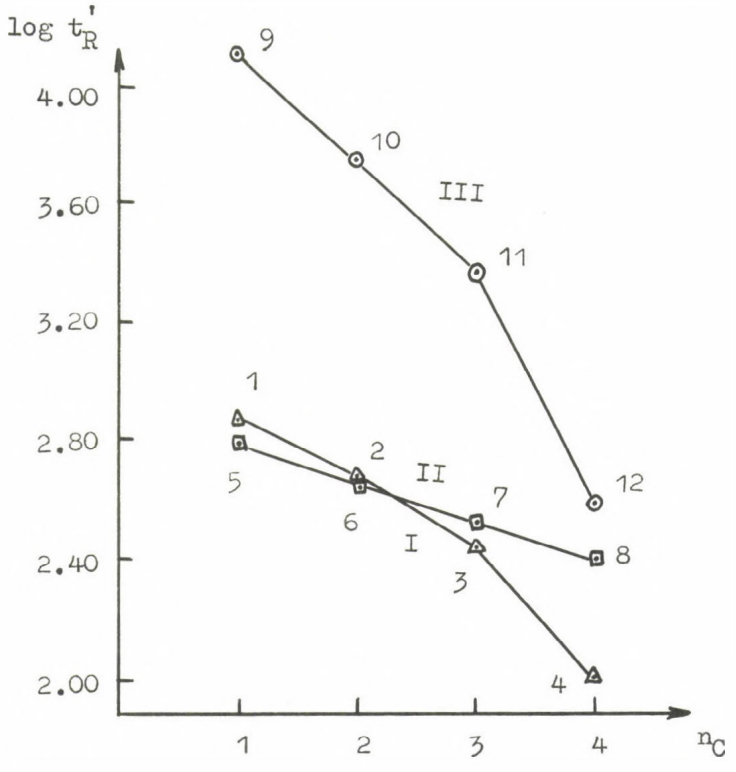
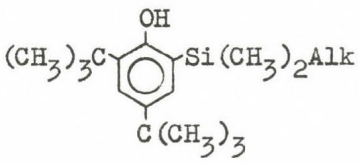


Fig. 1. Dependence of the logarithm of the adjusted retention time (log t_R') on the number of carbon atoms (n_C) in the alkyl substituents of the silicon-containing compounds. Roman and arabic numerals identify the compounds as in Table 1.

As we have reported in our recent paper [7], the retention times of silicon-containing phenols of the type



(where Alk = methyl or ethyl)

on Silasorb 600, using n-hexane as the mobile phase, under the same experimental conditions as used in this work, are only about 3 minutes. This is due to the steric hindrance of the phenol hydroxy group, playing the main role in the retention. However, as seen in Table 1, the retention times of the silicon-containing phenols investigated in this work (compounds 5-8), are 2-4 times as much. This is due to the decisive contribution of the specific interaction of the unshared electron pairs of the oxygen atom in the dimethylalkoxysilyl substituents with the protonated hydrogen atoms of the silanol hydroxy groups on the adsorbent surface. Analogous considerations may be made in respect of the compounds corresponding to Type I and III. Moreover, there are experimental data showing that compounds similar to Type I and III compounds, but having dimethylalkylsilyl ($SiMe_2Alk$) substituent instead of the dimethylalkoxysilyl ($SiMe_2OAlk$) substituent, are retained several times weaker on Silasorb 600. The above mentioned specific interaction is also mainly responsible for the retention of aryloxysilanes (Type I: compounds 1-4) and substituted disiloxydiphenyls (Type III: compounds 9-12), because in the molecules of these compounds other active sites, capable of influencing as much the retention on Silasorb as the oxygen atom of the dimethylalkoxysilyl group, are absent. Naturally, the specific interactions of the oxygen atom adjacent to the aromatic ring, and the π-electrons of the aromatic ring with the adsorbent surface also contribute to the adsorbate-adsorbent intermolecular interaction. However, the oxygen atom of the aryloxysilanes adjacent to the aromatic ring is hindered with a bulk $SiMe_2OAlk$ group; furthermore, the interaction of the π-electrons of the aromatic ring with the adsorbent surface is rather weak (e.g. under the conditions used by us, benzene is eluted in approximately two minutes), and is sterically hindered with three

bulk substituents (two tert.-butyl and one dimethylalkoxysilyl groups).

The rather close retention times of the corresponding compounds of Types I and II having the same alkyl substituents also indicate that the proposed active site in the molecules of the investigated silicon-containing compounds is the extent of the steric hindrance of this active site with the adjacent substituents, i.e. the steric effects of the alkyl substituents. Consequently, one may expect that there should be a correlation between the chromatographic retention data ($\log t_R'$) and the steric constants of the alkyl substituents. In order to investigate the existence of such relationships, we have plotted in Figs 2 and 3 the logarithms of the adjusted retention times of the investigated compounds against the Palm and Charton steric constants of the alkyl substituents present in these compounds.

Chumakov and Kabulov have shown [8] that for 2-alkylpyridines a linear correlation exists between $\log t_R'$ (determined by liquid chromatography on silica gel) and the Taft, Palm and Hancock steric constants of their alkyl substituents. As can seen from Figs 2 and 3, there is also a certain correlation between $\log t_R'$ values and the steric constants of the compounds investigated by us; the retention decreases when the steric constants of the alkyl substituents adjacent to the oxygen atom responsible for the main contribution to the retention increase. However, while a practically linear relationship exists between the $\log t_R'$ values and the steric constants of Palm ($E^o_{s(Alk)}$ and $E^o_{s(CH_2Alk)}$) (see Fig. 2), the relationship between $\log t_R'$ and the steric constants of Charton (ν) is more complicated (Fig. 3). The deviations from linearity seem to be caused by two reasons. First, the retention in HPLC (i.e. $\log t_R'$) is determined not only by the specific adsorbate-adsorbent intermolecular interaction, but also by the non-specific adsorbate-mobile phase intermolecular interaction. In our case, using n-hexane as the mobile phase, the latter increases when going from compounds with methyl substituent to compounds with tert.-butyl substituent. According

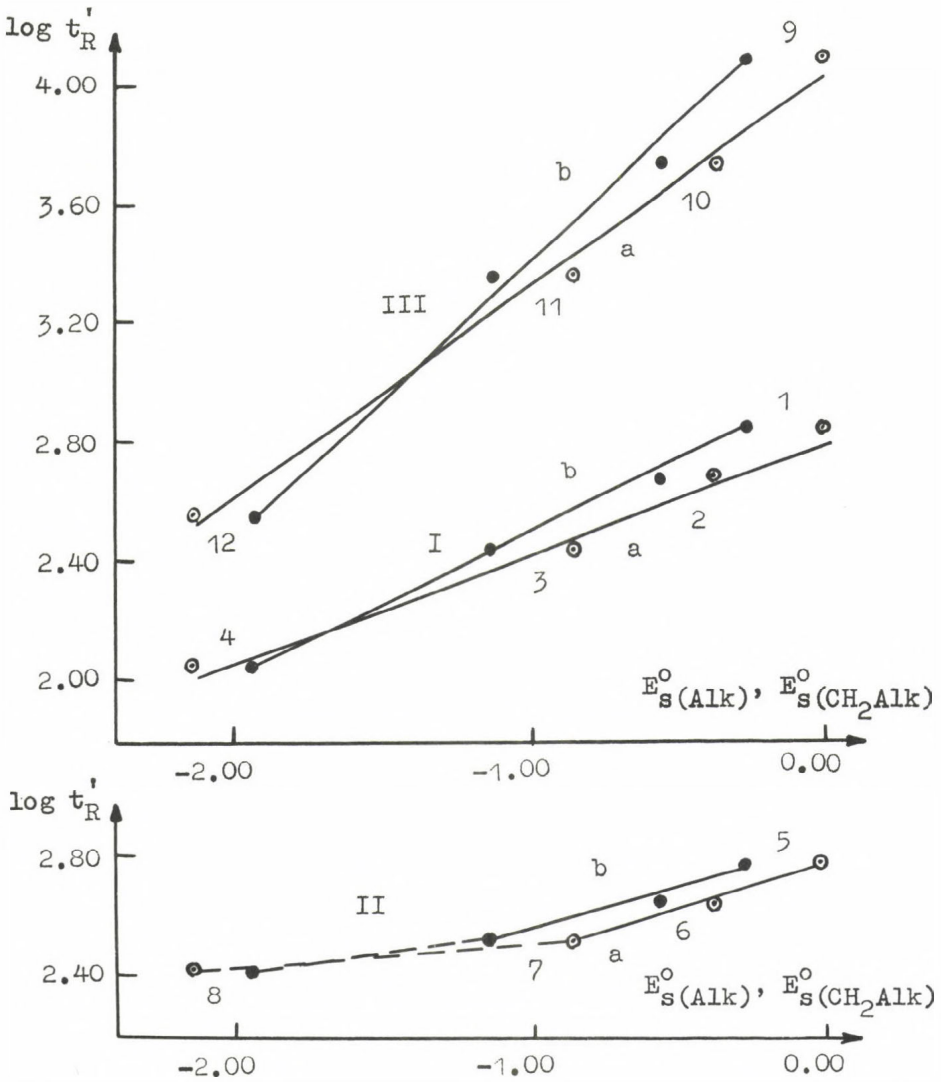


Fig. 2. Relationship between log t'_R and the steric constants of Palm [$E^o_{s(Alk)}$ and $E^o_{s(CH_2Alk)}$] for silicon-containing compounds. Roman and arabic numerals identify the compounds as in Table 1.

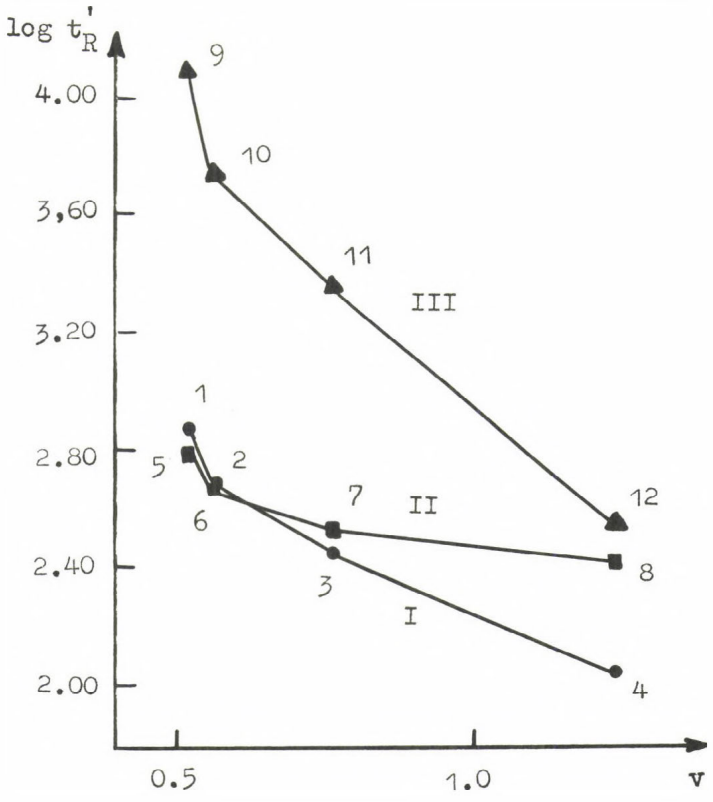


Fig. 3. Relationship between log t'_R and the steric constants of Charton (v) for silicon-containing compounds. Roman and arabic numerals identify the compounds as in Table 1.

to the second reason, the steric effects of the alkyl substituents seem to influence in different ways the adsorption of the investigated compounds on the adsorbent surface, and their interaction in the solution with the reference compounds used for the calculation of the steric constants. Nevertheless, a noticeable correlation exists between the log t'_R values and the steric constants of the alkyl substituents of the investigated silicon-containing compounds. One may hope

that the consideration of the non-specific adsorbate-mobile phase intermolecular interaction, as well as the utilization of data for a greater number of compounds, with both linear and branched alkyl substituents, will make it possible to establish a quantitative relationship between the HPLC data and the steric constants of the substituents. This will permit to use HPLC as a rapid and convenient method for the determination of the steric constants of the substituents. We continue our investigations in this field.

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SPECIFIC SORBENTS FOR CHROMATOGRAPHY OF NUCLEOLYTIC ENZYMES

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The continuously rising demand for pure enzyme preparations of microbial origin is stimulating the development and perfection of methods for their isolation and purification. For this purpose specific sorbents containing nucleotides, amino acids, lipids and other compounds are used increasingly as stationary ligands.

The present paper describes the isolation of exonuclease A5 by affinity and ligand-exchange chromatography. This enzyme cleaves RNA and denatured DNA, acting from the 3'-end of the molecule. It is not specific with respect to the sugars of the nucleic acids and the structure of the heterocyclic base; likewise has no appreciable influence on the rate of hydrolysis /1, 2/. Exonuclease A5 has proved to be a useful reagent both for investigating nucleic acids /3/ and for solving practical problems, such as hydrolysis of the total yeast RNAs to 5'-nucleotides, which are intensifiers of the taste and smell of food products /4/.

We have previously investigated competitive inhibitors of the type of pyrimidine nucleoside 2' (3'), 5'- diphosphates (pNp) with various modifications in the heterocyclic base and the carbohydrate moiety of the molecule /5/. For the compounds investigated, the values of K_i proved to be close to one another at 10^{-5}M, which permits using them as stationary ligands for affinity chromatography.

It has been established /6/ that the introduction of a substituent (R) into the 5'-phosphate residue, i.e., the conversion of pNp into RpNp sharply diminishes the inhibiting

capacity of the compound, while the introduction of a substituent into the 3′-phosphate group with the formation of pNpR makes the compound a substrate if R has a nucleoside nature. Consequently, we considered it desirable to add the inhibitor to the support through the heterocyclic base.

It is knownthat uridine having electron-donating substituent in position 5 is capable of reacting with aryldiazonium salts, forming 6-arylazouridine derivatives. We have used this property for the addition of nucleotide ligand to aminoaryl-containing sorbents with the aid of the azo coupling reaction. With this end in view we have synthesized 5-hydroxyuridine 2′ (3′), 5′-diphosphate /7/.

For the immobilization of the ligand we selected aminoaryl containing organosilicon sorbents based on Silochrome. The Silochrome was previously treated with aluminium salts to increase its stability in aqueous solutions /8/. To suppress the non-specific sorption of proteins, the Silochrome treated with aluminium salts (Alusil) was coated with organic polymers. In the present investigation we used two organosilicon sorbents containing 5-hydroxyuridine 2′ (3′), 5′-diphosphate as the stationary ligand (Fig. 1). Sorbent I was obtained from Alusil after preliminary treatment with 3-(2′, 3′-epoxypropoxy) propyltrimethoxysilane /7/. The epoxide ring was opened in an acidic solution of acetone, the aminoaryl derivative was obtained with the aid of p-nitrobenzoyl chloride followed by reduction of the nitro group. Sorbent II was obtained with the aid of addition of 3.5% poly (γ-hydroxymethylmethacrylate) to Alusil /9/ with subsequent conversion of the hydroxy groups into aminoaryl-groups and coupling 5-hydroxyuridine 2′ (3′), 5′-diphosphate /7/. In order to protect the ligand nucleotides from the action of the phosphomonoesterase present in the preparation they were converted into methyl esters. The methanolysis reaction was performed on the sorbents obtained.

The scheme of purification for the exonuclease A5 is shown in Table 1. The impurities in the 5′-exonuclease preparations that are the most undesirable and difficult to eliminate are non-specific phosphatase and 5′-nucleotidase. In the initial preparation with which we worked, the ratio of the nuclease and

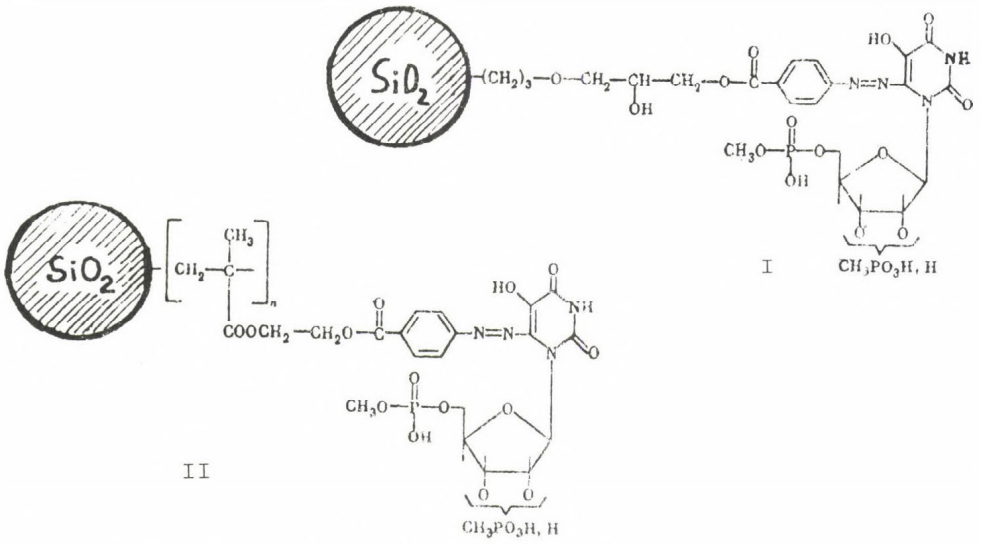

Fig. 1. The structures of affinity sorbents

Table 1. Scheme of Purification of Exonuclease A5

Stage of purification	Total nuclease activity, activity units (act. un.)	Specific nuclease activity, activity units mg of protein	N/P ratio	Yield %
Initial preparation	80 000	1310	2.5	100
Salting-out of total protein with ammonium sulfate	47 360	5980	3.3	59
Gel chromatography on Sephadex G75	41 900	6120	10.2	52
Affinity chromatography (0.5 g of sorbent)	21 220	96000	212	27

phosphatase activities (N/P) was 3. Preliminary purification was effected with the aid of salting-out and gel chromatography. Buffer A (5mM Tris-HCl, pH6.8, containing 100 mM NaCl and 1 mM $MgCl_2$) which is necessary for binding the enzyme with the bio-

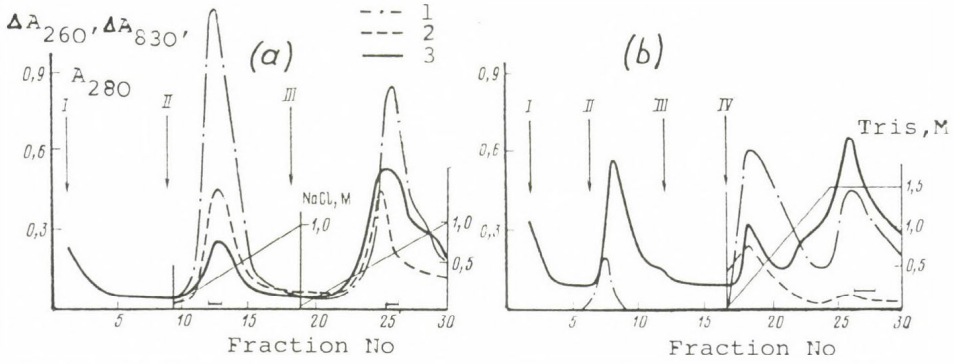

Fig. 2. Affinity chromatography of exonuclease A5 on sorbent I. Column 0.8x2 cm, rate of elution 17 ml/h, fraction volume 8.5 ml. 1) exonuclease activity, which is proportional to A_{260}, 2) phosphatase activity, which is proportional to A_{830}, 3) protein content, A_{280}. The arrows show changes in the eluents: (a) I-buffer A, II-linear gradient of NaCl (100 mM-1 M) in 50 mM Tris-CH_3COOH buffer, pH 5, containing 5 mM $MgCl_2$, III-linear gradient (50 mM-1 M) of Tris-CH_3COOH buffer, pH 5, containing 5 mM $MgCl_2$, (b) I-buffer A, II-20 mM Na_2HPO_4 in buffer A, III-buffer A, IV-linear gradient (50 mM-1.5M) of Tris-CH_3COOH buffer, pH 5, containing 5 mM $MgCl_2$. The fractions collected for subsequent analyses are indicated

specific sorbent I, was used for elution from Sephadex G75. When sorbent II was used in the last stage a preparation with similar characteristics was obtained: 60 000 activity units mg of protein and N P ratio 260 /7/.

It was shownearlier that under these conditions nonspecific sorption on the sorbent without the ligand amounted to 0.5 mg of protein g, and this value fell to zero if the sorbent was re-used. The biospecific sorbent was fairly stable in use: after 10 cycles of working in a column the sorbent retained 98% of the stationary ligands.

When the biospecific sorbent was saturated with the enzyme preparation, it was washed with buffer A until the eluate showed no absorption at 280 nm. Unfortunately, not only the nuclease but also the phosphatase was sorbed on the column, and therefore it was necessary to select conditions for their separation. Several different types of elution were investigated. On gradient elution (Fig. 2a), the first peak corresponded to a preparation with a N/P ratio = 30. Under these conditions a considerable proportion of the nuclease activity remained in the column, and it was possible to remove it only with the aid of a buffer having a high (1 M) concentration of aminotris (hydroxymethyl) methane.

The possibility of specific elution of the phosphatase was investigated by using solutions of its substrates and inhibitors such as p-nitrophenyl phosphate, fructose 1,6-diphosphate or disodium phosphate. Preparations with the best N/P ratio were obtained by using a 20 mM solution of Na_2HPO_4 in buffer A (Fig.2b). At the same time the nuclease was partially eluted together with the phosphatase, which confirms the difficulty observed previously /9/ in the chromatographic separation of these enzymes with the use of the culture grown, as in our case, in the presence of $CaCO_3$. After the removal of the phosphatase bulk by elution with a 20 mM solution of Na_2HPO_4,

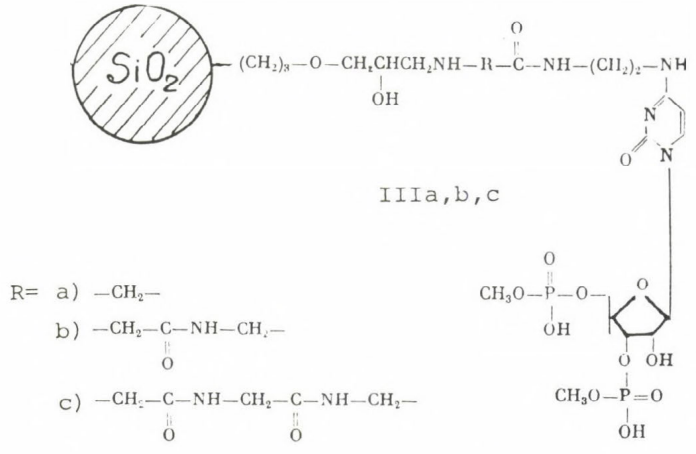

Fig. 3. The structures of affinity sorbents

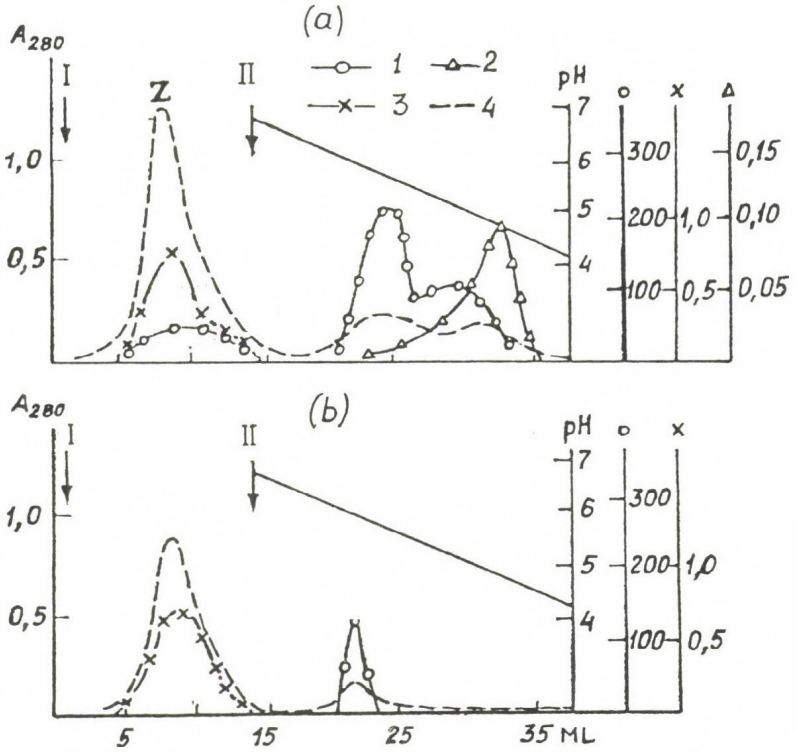

Fig. 4. Ligand-exchange chromatography of exonuclease A5 on Ni^{++}-iminodiacetic acid coupled Toyopearl HW-55. Column 0.8x2 cm, rate of elution 10 ml/h. (a)-First chromatography. (b)-Rechromatography of fraction Z. 1) Exonuclease activity, act. un./ml, 2) phosphatase activity, act. un./ml, 3) 5'-nucleotidase activity, act. un./ml, 4) protein content, A_{280}. The arrows show changes in the eluents: I-5 mM Tris HCl, pH6.8, containing 1M NaCl (buffer b), II-linear gradient of pH: buffer B buffer C(5 mM NH_4OAc, pH4.2, containing 1M NACl)

gradient elution was used. The last peak, eluted by 1.5M Tris-CH_3COOH buffer, pH5, corresponded to a preparation with an N/P ratio of 780. The electrophoresis of exonuclease A5 in a 9% polyacrylamide gel at pH8.9 in the presence of denaturing agents (sodium dodecyl sulfate and 2-mercaptoethanol) gave two protein zones: one distinct and intense, the other weak.

We also synthesized other types of organo-silica affinity sorbents /10/. Those sorbents were obtained by attaching N^4-(aminoetyl) cytidine-2'(3'),5'-diphosphate through a hetero-

cyclic base to Silochrome, coated with polyacrylic acid or treated with 3(2',3'-epoxypropoxy) propyltrimethoxysilane. A number of sorbents showed different spacer arms, such as glycine, diglycine or diglycylglycine, between the ligand and support (Fig. 3). The exonuclease A5 preparation that we purified by means of those sorbents according to scheme in Table 1 has an N/P ratio of 3000.

Another method which we used in order to purify exonuclease A5 was ligand-exchange chromatography. Ligand-exchange support based on Silochrome and TSK-Gel Toyopearl HW-55 with iminodiacetic acid as stationary ligand were syntesized. The efficacy of the TSK-Gel HW-55 based chelating sorbent (Ni^{++}-form) for purification of exconuclease A5 is demonstrated (Fig. 4.). The exonuclease A5 preparation that we purified by means of ligand-exchange chromatography do not contain impurities such as nonspecific phosphatase and 5'-nucleotidase, and are homogeneous in polyacrylamide gel /11/.

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THE PREPARATION OF RP SILICA; SILYLATION TEMPERATURE AS A MEANS FOR ADJUSTING THE CHROMATOGRAPHIC PROPERTIES

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INTRODUCTION

For describing the retention on RP silica the nature and surface concentration of bonded alkyl groups are often stressed. On the other hand, selectivity, peak shape and chemical stability are also strongly influenced by the nonsilylated residual silanol groups /1/. Only a few papers have been published (e.g. /2 - 4/) dealing with investigation of the silylation parameters and silanol concentration. Increasing the reaction temperature was found to be a powerful tool to deactivate surfaces of glass capillaries /5/ and silica /6/.

The role of temperature during silylation is investigated in detail. In our experiments only di-n-alkyl-tetramethyldisilazanes were used, because they show a high thermal stability and act as monofunctional silylating agents yielding a bonded monolayer. Ammonia evolved during the reaction can act as a catalyst and supports the rate and degree of modification /7/. A silica modified by such reagents shows higher efficiency and facilitates further calculations.

EXPERIMENTAL

The silylation procedure used has been described earlier /6/. Essentially, the dehydrated silica samples were modified at room temperature for 30 minutes or at a temperature of 139^{o}C

for 8 hours in m-xylene. At higher temperatures the reaction was carried out in a glass ampoule.

LiChrosorb Si 100, 10 µm (E. Merck, Darmstadt, F.R.G.) of the same batch was used in all experiments. This material was characterized by a specific surface area (S_{BET}) of 257 $m^2 g^{-1}$ and a very narrow and symmetrical distribution of the pore size with a mean value ($\bar{d}_p$) of 15 nm. In this case the determination of the surface silanol group concentration by the methyllithium method /8/ gives correct and reproducible values, because the solvated reagent can penetrate into all pores /9/. The silanol group concentration (α_{OHo}) of this starting silica was determined to be 9.0 µmole m^{-2}. Elemental analysis gave the surface concentration of organic groups (α_C) in µmol m^{-2} calculated according the formula of Berendsen et al. /10/.

The chromatographic investigations were carried out in a micro HPLC apparatus /11/. Glass-lined tubing 150 x 0.6 mm I.D. (SGE, Australia) served as the column material. Water-saturated, analytical grade n-heptane (VEB Berlin-Chemie, Berlin, GDR) was used as the eluent.

The following disilazanes /5/ were used: tetramethyldisilazane (HMDS), di-n-buthyltetramethyldisilazane (DBTMDS), di-n-hexyltetramethyldisilazane (DHTMDS), di-n-octyltetramethyldisilazane (DOTMDS), di-n-decyltetramethyldisilazane (DDTMDS) and di-n-octadecyltetramethyldisilazane (DODTMDS).

RESULTS AND DISCUSSION

Silylation temperature and surface groups

The resulting concentrations of the surface groups, (α_C and α_{OH}) obtained by the chemical modification of silica at different temperatures with HMDS, DHTMDS and DDTMDS are given in Figs 1, 2 and 3, respectively. The plots of the surface concentration of organic groups, represented by □, vs. the reaction temperature exhibit for each disilazane a curve with a broad maximum. The maximum α_C values are about 5 µmole m^{-2} for RP-1 and RP-6, and somewhat lower (3.5 µmole m^{-2}) for RP-10.

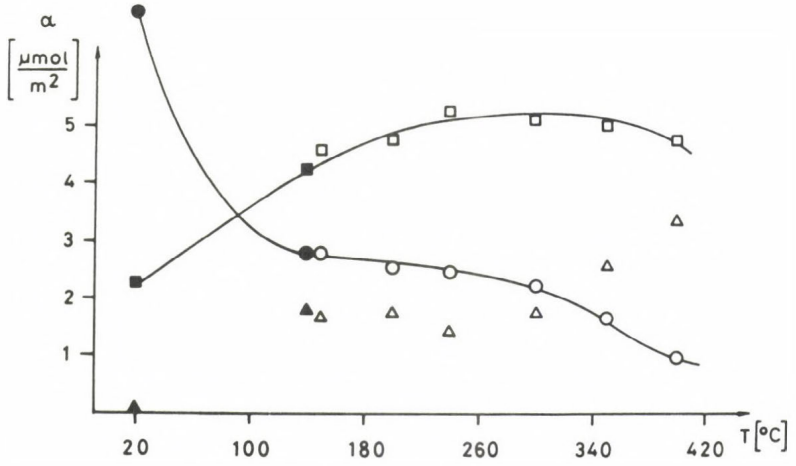

Fig. 1. Dependency of the surface group concentrations on the silylation temperature using HMDS as the reagent. □ , ■ organic groups; o, ● silanol groups; △, ▲ Δαvalues; open symbols for vapour phase reaction, full symbols for reaction in m-xylene

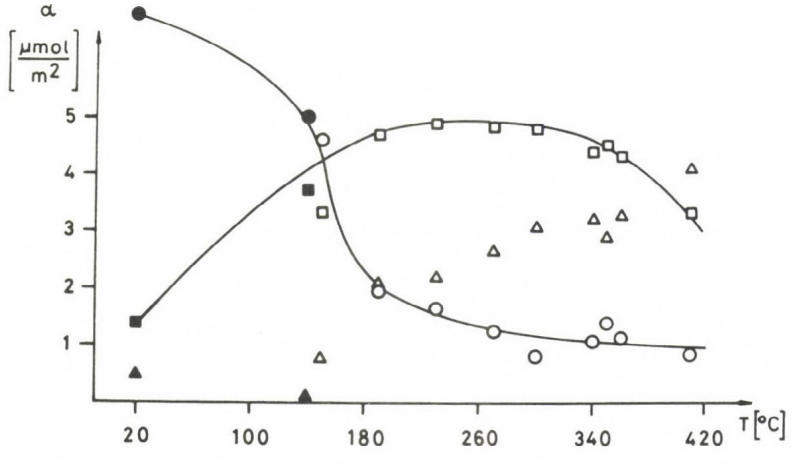

Fig. 2. Dependency of the surface group concentrations on the silylation temperature using DHTMDS as the reagent. Symbols as in Fig. 1

This confirms the fact that only about 50% of the silanol groups of the parent silica can be converted.

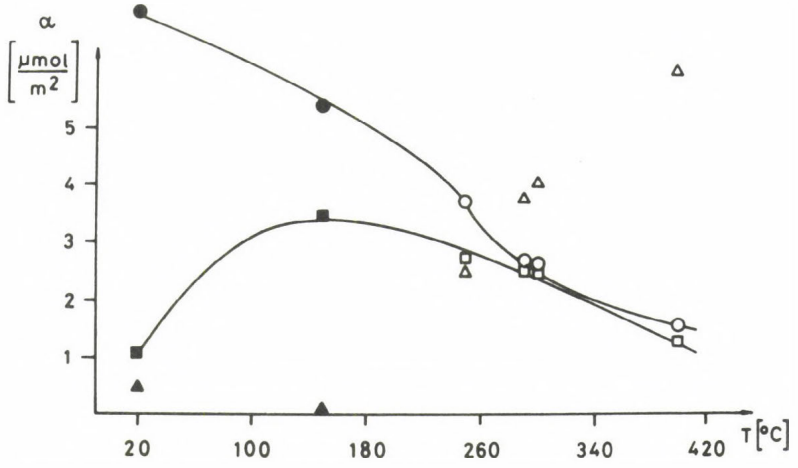

Fig. 3. Dependency of the surface group concentrations on the silylation temperature using DDTMDS as the reagent. Symbols as in Fig. 1

Sterical hindrance was stated as a reason for this /12/. At lower temperatures the modification is incomplete yielding smaller α_C values. At higher temperatures a decrease of the surface covering can also be seen. An explanation might be the interruption of the Si - O - Si R^3 bond. According to Berendsen et al. /10/, van de Venne /13/ and Hansson and Trojer /14/ this interruption starts at temperatures between 250 and 300°C. Trojer and Hansson /15/ proved by means of pyrolysis gas chromatography that during capping processes octadecyl groups are partly exchanged by methyl groups. As obvious from Figs 1, 2 and 3 the ratio of linking and interrupting the Si - O - Si R^3 bonds depends on the temperature and the chain length of the reagent used yielding different maxima and descending parts of the curves.

The concentrations of residual silanol groups, represented by o, decrease with increasing temperature and approach asymptotically a value of about 1 μmol m^{-2}. This behaviour cannot be explained by thermal dehydroxylation only /6/. It was found that the reduction in silanol concentration (α_{OH}) is not accompanied by an equal increase of the organic group surface

concentration (α_C). This led us to introduce the $\Delta\alpha$ value, representing the difference between the surface concentration α_{OHo} in the starting material (9.0 µmole m^{-2}) and the sum of α_{OH} and α_C in the modified silica, representing a new surface element, which was assumed to be a siloxan bridge /5, 6/. At lower temperatures up to about 150°C the $\Delta\alpha$ values are scattered around zero. With increasing temperature the values increase up to about 5 µmole m^{-2}.

These explanations are supported by the results of the chemical modifications with DBTMDS (RP-4), DOTMDS (RP-8) and DODTMDS (RP-18).

The reproducibility of the silylating process is good, as demonstrated by the 3 RP-6 materials (Fig. 2) prepared at temperatures of about 350°C.

These experiments show that by using the full temperature range up to the decomposition of the reagent RP materials with tailor-made properties can be produced: low or maximum covering with organic groups and/or high or strongly reduced content of residual silanol groups.

Chromatographic indication of silanols

The methyllithium method used for the determination of the surface concentration of the residual silanol groups has some drawbacks. It gives erroneous findings for small pore silicas /9/ and is not practicable for materials in prepacked columns. Therefore a chromatographic value has been proposed for the characterization of silanols. Often the fixed retention value of a polar compound such as nitrobenzene using n-heptane as the eluent is used to distinguish between well and poorly modified silicas /16 - 18/.

In this work we tried to correlate the α_{OH} values determined by the methyllithium method with the capacity ratios (k') to find a semi-quantitative measure for the presence of silanols. Several aromatic compounds representing the different types of interaction with silanol groups are chromatographed in n-heptane saturated with water. As an example the lg k' values of nitrobenzene, aniline and benzyl alcohol are plotted vs. the α_{OH}

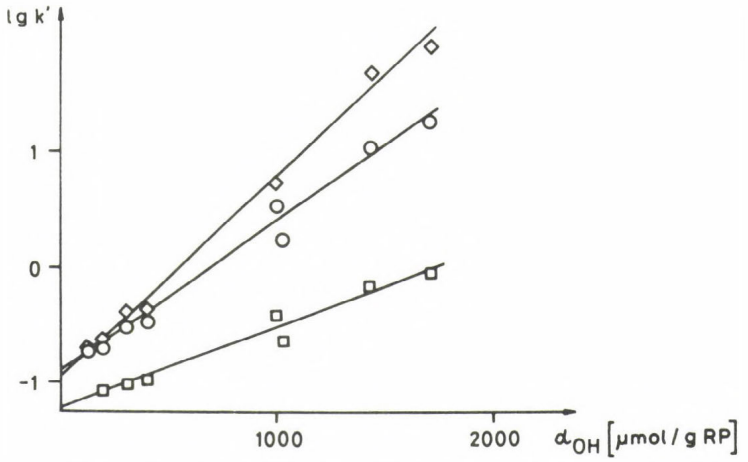

Fig. 4. Plot of the lg k' values vs. the silanol concentration of different RP-6 materials. Eluent: Water-saturated n-heptane; Temperature: 22°C; □ nitrobenzene; o aniline; ◊ benzyl alcohol

values of different RP-6 materials (Fig. 4). It is obvious that linear dependencies exist. The intercepts with the y-axis should be very small according to the low hydrophobic interaction in this chromatographic system /17/. Differences may result from the estimation of the dead time with n-hexane as the unretained compound. The steepest slope can be seen for benzyl alcohol, possibly due to the ability of forming strong hydrogen bonds. Surprisingly aniline shows only a medium slope among these compounds. The range of the lg k' values of nitrobenzene is relatively small. This suggests that nitrobenzene is not the most suitable indicator for silanol groups. It gives less information especially in the lower range of silanol concentrations /6/.

Similar linear dependencies were found for all RP materials examined, but the slopes significantly differ.

The influence of the chain lengths of the bonded organic groups on the chromatographic effect of the silanols is illustrated in Fig. 5. (For purposes of comparison, materials with a silanol surface concentration of 1000 µmole m^{-2} were taken from plots such as Fig. 4). The smallest k' values may be seen

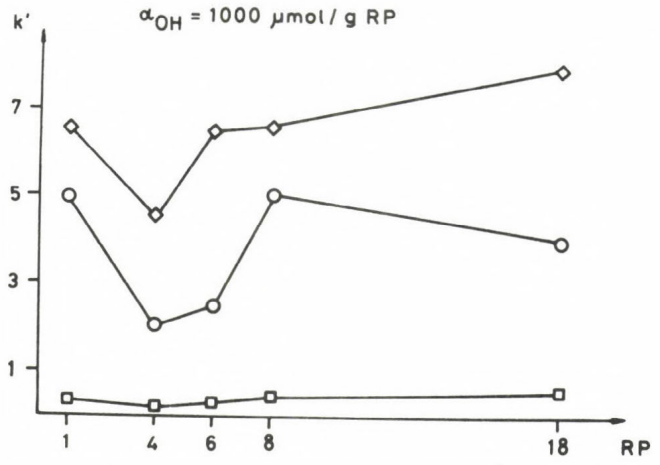

Fig. 5. Influence of the chain length of the bonded organic groups on the retention (k') for materials with constant silanol group concentration. Conditions and symbols an in Fig. 4

on the RP-4 material, a fact also observed by Karch et al. /16/ and attributed to the superior shielding effect of the C_4-bristles. As a consequence of the semi-quantitative interpretations of such k' values one has to take into consideration the nature of the bonded alkyl groups. The criterion $\lg k'_{\text{nitrobenzene}} < 0.5$ for well-deactivated RP materials /16 - 18/ seems to be rather arbitrary.

Chromatographic tests of RP materials basing on silica with a pore diameter of 10 or 6 nm indicate a residual silanol concentration in the expected range according to the modification conditions. This means that the described method can supply valuable information. A paper containing the detailed slopes for different RP materials and all test compounds used is in preparation.

Two examples for choosing appropriate RP materials to solve real analytical problems are given below. The first example (Fig. 6) shows the separation of the reaction mixture of N-methallyl-N-tosyl-p-toluidine and sulfur dichloride on a weakly silylated RP-4 material using n-heptane as the eluent. This separation could not be achieved in the classical RP mode due to the insolubility of the sample in solvents such as methanol

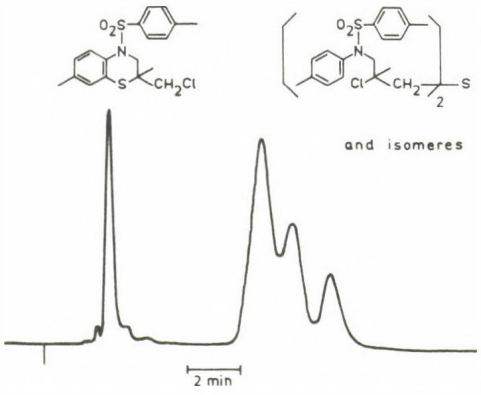

Fig. 6. Separation of the reaction mixture of N-methallyl-N-tosyl-p-toluidine and sulfur dichloride. Column: Glass-lined tubing 150 x 0.6 mm I.D.; Packing: RP-4, 10 µm, α_{OH} = 1200 µmole g^{-1} RP; Eluent: Water-saturated n-heptane; Flow rate: 50 µl min^{-1}; Detection: 254 nm

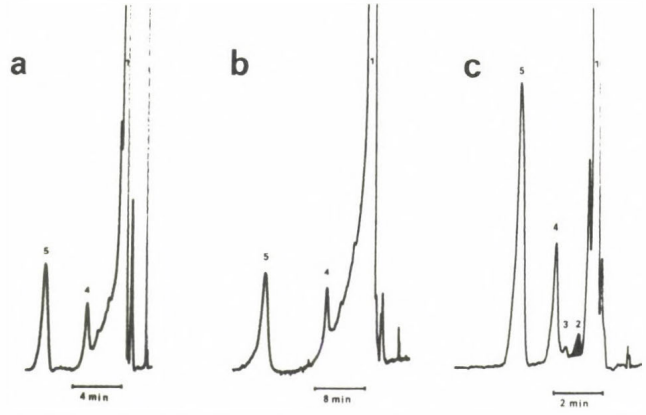

Fig. 7. Separation of an enzymatic reaction mixture. Column, packing and pressure, respectively: a) 200 x 4.6 mm, RP-8, 10 µm, commercial product, 6 MPa; b) 250 x 4 mm, RP-8, 10 µm, commercial product, 10 MPa; c) 150 x 4 mm, RP-6, 10 µm, α_{OH} = 260 µmole g^{-1} RP, 3.2 MPa; Eluent: CH_3OH/H_2O, 35/65 (v/v); Detection: 226 nm; Compounds: 1 dimethylformamide, 2 Ac-Phe-Ala-NH_2, Ac-Phe-Ala-OH, 4 Ac-Phe-OH, 5 Ac-Phe-OMe

or acetonitrile. On the other hand, in the adsorption mode no elution could be obtained on pure silica and no separation could be accomplished on well-silylated RP material.

The advantageous application of RP material with low silanol concentration is illustrated in Fig. 7. In comparison to commercial RP materials this RP-6 column offers smaller k' values and better peak shape. Therefore one interesting compound (peak 2), disappearing below the tailing of the solvent on the commercial products, can be well separated in a shorter time.

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DETERMINATION OF SOME BED AND SORBENT CHARACTERISTICS FROM STREAMING CURRENT RESPONSES

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SUMMARY

Elution volume of the first streaming current response, detected at a suitable mobile phase composition in normal-phase systems before the column dead volume, is in an exact accord with the exclusion volume of the column. The elution volume corresponding to the vacant streaming current response of different silica gels, registered closely to the column dead volume, is between the column dead volume and the total volume of the liquid phase in the column. Elution volumes of these streaming current responses can be used not only for the determination of the exclusion and dead volumes of the column but also for the determination of the mobile phase volume in the particles of the packing, of external and internal porosities, total bed porosity and the specific pore volume of silica gel, or also for the determination of the mean pore diameter of silica gel in the bed and of the specific weight of its skeleton.

INTRODUCTION

Origin of the streaming current during the flow of the liquid along the solid surface has been known for a long time. However, this phenomenon has been used only recently for detection purposes in liquid chromatography [1-7]. In studies on the generation of the streaming current in the bed of a polar sorbent through which a mobile phase of low polarity flew which had a low relative permittivity and low conductivity, it was observed that in addition to the responses corresponding to the elution of the injected solutes additional responses [8,9] also originated in these systems.

Comparing records obtained by the registration of the streaming current with the response of common detectors, e.g., refractometer or photometer, we found that the first streaming current response was eluted in a considerably smaller mobile phase volume than it corresponded to the column dead volume (Fig. 1). In the chromatogram obtained with refractometric or photometric detection we never succeeded in finding the response eluted in the same volume of the passed mobile phase. The elution volume of the first streaming current response agrees with the exclusion volume of the column [9]. This exclusion response originates at the injection of any solute with a syringe. It is generated also when a needle or other solid body is introduced into a mobile phase stream or when the mobile phase starts flowing through the column. It is registered even with the injection of several microlitres of the mobile phase taken from the chromatograph with a syringe, introduced via the septum into the injector, and injected backwards. Causes of the origin of the exclusion volume response have not yet been successfully explained.

The second streaming current response originates when we work with multicomponent mobile phases in which at least one of polar components is present in concentrations up to tens of percents. The origin of the streaming current response in a binary mobile phase was ascribed to the vacancy of the polar mobile phase component eluted in the dead volume [9].

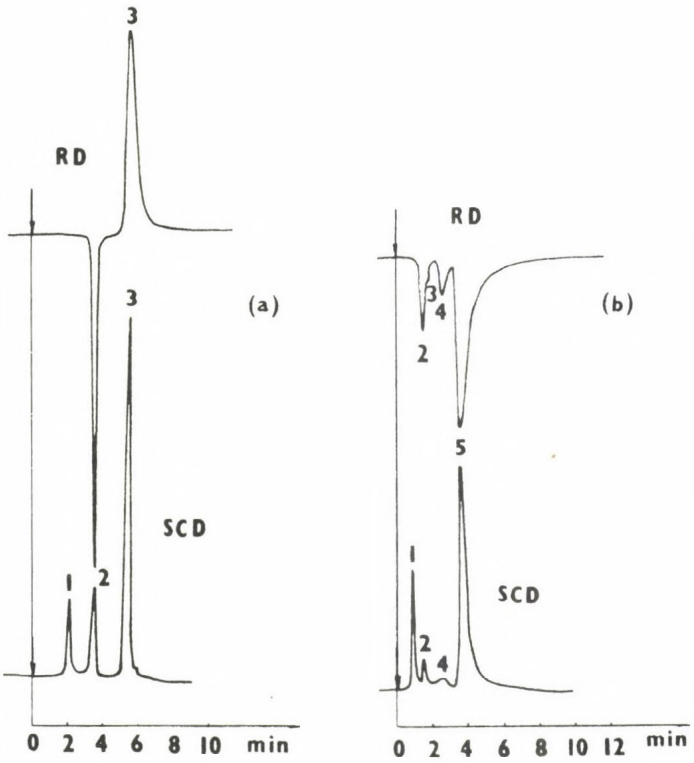

Fig. 1
Typical records with refractometric detection (RD) and streaming current detection (SCD) after the injection of
(a) the solute in the polar component of the mobile phase and
(b) the pure liquid solute
Stationary phase: Silasorb 300, d_p = 10 /um, column: 200x4 mm.
Mobile phase: 95:5, heptane - isopropanol.
(a) Injected volume: 4 /ul, mobile phase flow rate: 0.65 ml/min.
Peaks: 1 - exclusion streaming current response, 2 - vacant response, 3 - p-nitrophenol
(b) Injected volume: 2 /ul, mobile phase flow rate: 1.00 ml/min.
peaks: 1 - exclusion streaming current response, 2 - vacant response, 3,4 - impurities, 5 - methanol

The existence of these streaming current responses offers possibilities for a rapid and easy determination of some characteristics of the chromatographic bed and even of the used sorbent. Some of these were already mentioned in ref. [9]. Accuracy and precision of these determinations are, however, dependent on two basic assumptions:

i) The elution volume of the first streaming current response can be considered as an exclusion volume of the column;

ii) The second streaming current response is caused by the vacancy of the polar mobile phase component eluted in the dead volume of the column.

We, therefore, decided to prove the accuracy of these assumptions. New experience and ideas on the measurement of the column dead volumes were considered in this connection. On the basis of the results obtained in this way, the possible use of the exclusion and vacant streaming current responses was evaluated. For experimental proof we chose hydrothermally modified Silasorb type silica gels and several commercial silica gels chosen at random.

EXPERIMENTAL

A Varian 8500 pulseless pump (Varian, U.S.A.) served as the source of the mobile phase flow. A Variscan spectrophotometer (Varian, U.S.A.) and a Knauer 2025/50 refractometer (Knauer, FGR) both having cells with 8 µl volume were used for detection. Stainless-steel columns, 6x200 mm, were filled by a viscosity variant of the high-pressure filtration technique [8]. The minimum reduced height equivalents to a theoretical plate of the unsorbed substance, calculated for certified particles, varied over the range of h = 1.9-2.3. Silica gel samples were packed into the column tube either as obtained from the manufacturers or after a hydrothermal treatment. Microlitre volumes of the sample solutions were injected into the mobile phase stream with injection syringes.

Specific surface areas were measured by the dynamic nitrogen thermal desorption method [10] using a standard

(Al_2O_3, S =,203 m^2/g). Pore volumes were determined by direct titration with water [11,12]. The amount of the sorbent in the column was determined by weighing of the empty and the filled column after elimination of the butanol mobile phase.

Hydrothermally modified silica gels [13,14] were prepared as research samples from a coarser fraction of the commercial silica gel Silasorb 500 (Lachema, Brno, Czechoslovakia). The following commercial packings, selected at random, were also used: Nucleosil 100-10, batch 9111 (Macherey-Nagel, FGR), Hypersil, batch 80.S.03 (Shandon Southern Products, Great Britain), Separon SI-VSK, batch SG-12 (Laboratory Instruments, Prague, Czechoslovakia) and two products of E. Merck (Darmstadt, FGR) - LiChrospher Si 100, batch VV696 and LiChrosorb Si 60, batch EH8. More detailed data on the used silica gels are presented in Table 1.

Analytical grade n-hexane (Soyuzkhimexport, USSR) with the addition of 0.7 or 0.05 vol %, of ethanol for UV (Lachema, Brno, Czechoslovakia) was used for the measurements with the refractometer or the photometer. For the streaming current detection 5 % of analytical grade butanol (Lachema, Brno, Czechoslovakia) were added instead of ethanol. The specific weight of this mobile phase, determined pycnometrically, was 0.667 g/ml. Analytical grade n-octane and squalane (Loba-Chemie, Austria) and analytical grade n-butanol served as the solutes for the measurement of the dead volumes.

The packed columns were stabilized with 250 ml of ethanol which then was removed from the bed with the mobile phase containing 0.7 % of ethanol. Prior to measurement with the mobile phase containing 0.05 % of ethanol the column was washed with 250 ml of freshly distilled, dried acetone. The butanol mobile phase was removed from the column at 160-190 ^{o}C with a stream of dried nitrogen. Equilibration of the columns with mobile phases was followed by the injection of a model mixture of the retained solutes. Chromatographic measurements were performed at room temperature. The volumes of the connections between the column and the measuring cell of the refractometer or the photometer were within the limits of experimental error of the actual dead volume of the columns.

Table 1. Characteristics of the sorbents

Sorbent	Shape[a]	d_p (/um)	Modif. anion	S[c] (m^2/g)	V_p (ml/g) sp.[b]	V_p (ml/g) H_2O[d]	V_p (ml/g) Eq.5[e]	V_p (ml/g) Eq.7[e]	D (nm) sp.[b]	D (nm) H_2O[f]	D (nm) Eq.10[g]
Nucleosil	sph.	10	-	377	1.0	1.00	0.68	0.64	-	10.6	7.2
Hypersil	sph.	5	-	196	-	0.73	0.65	0.59	10.0	14.9	13.3
Separon	sph.	10	-	465	1.5	1.54	1.48	1.36	-	13.2	12.7
LiChrospher	sph.	10	-	536	1.2	1.26	1.17	1.23	-	9.4	8.7
LiChrosorb	irr.	10	-	592	-	0.78	0.69	0.73	6.0	5.3	4.7
Silasorb	irr.	7.5	-	520	0.75	0.75	0.71	0.64	-	5.8	5.5
Q 15 - 2AW	irr.	15	F^-	448	-	0.72	0.56	0.56	-	6.4	5.0
Q 15 - 6AW	irr.	15	F^-	291	-	0.70	0.57	0.61	-	9.6	7.8
Q 15 - 10AW	irr.	15	F^-	189	-	0.70	0.60	0.62	-	14.8	12.7
P 129 - 3AW	irr.	15	F^-	286	-	0.70	0.62	0.63	-	9.8	8.7
P 129 - 5AW	irr.	15	$H_2BO_3^-$	293	-	0.72	0.56	0.58	-	9.8	7.6
P 129 - 7AW	irr.	15	CO_3^{2-}	286	-	0.72	0.58	0.63	-	10.1	8.1
P 129 - 8AW	irr.	15	PO_4^{3-}	294	-	0.72	0.58	0.61	-	9.8	7.9

a) sph. = spherical, irr. = irregular particles;

b) specified data for Nucleosil, Hypersil, LiChrospher and Lichrosorb are from ref. [15] while data for Separon and Silasorb represent manufacturers' specifications;

c) measured by thermal nitrogen desorption; d) V_p measured by water titration;

e) V_p calculated according to the given equation; f) D calculated from Eq.(9) using V_p determined water titration; g) D calculated from Eq.(10).

When the streaming current is sensed directly from the column, the measured elution volumes are not affected by the volume of the connections [2].

Origin of the Second Streaming Current Response

Low polar hydrocarbon-based mobile phases suitable for the generation of the streaming current must contain several volume percents of highly polar organic component, e.g. alcohol. The measurements summarized in [8,9] and some other, yet unpublished, investigations suggest that, in such mobile phases, the elution volume of the second streaming current response is fairly constant for a given column. The response of the refractometer or the photometer is also registered in the same mobile phase volume. Elution volumes of all three responses agree, within the measurement error, with the elution volume of the polar component of the mobile phase.

If we consider the knowledge obtained in the study of the so-called vacancy or system responses (see e.g., refs. [16-18]), we can confirm the preceding conclusion [9] that the second streaming current response, recorded after every injection of the sample containing retained solutes, is caused by the vacant zone of the polar component of the mobile phase. From the theory of the origin of streaming current [1,6] it follows that the change in the relative permittivity of the mobile phase evoked by the vacant zone is the actual cause of the response. This conclusion was confirmed by continuous measurements of the relative permittivity and conductivity of the mobile phases at the column outlet by the procedure described in ref. [8].

Elution Volumes of Streaming Current Responses

The physico-chemical aspects of the determination of the column dead volume have recently been discussed. It was demonstrated that in chromatographic systems with a multi-component liquid mobile phase an unambiguous instruction for column dead volume measurements cannot be derived on thermodynamical basis [19,20]. Therefore, the problem can

only be solved by the introduction of a conventional procedure. Using reversed-phase systems as the example, Melander et al. [21] showed that the following definition is the most useful from a chromatographic point of view: In a given chromatographic system and under given experimental conditions, the column dead volume, V_M, can be considered to be identical to the elution volume of the least sorbed component of the mobile phase. The same convention has commonly been used in normal phase systems since the origin of HPLC. In such systems, the column dead volume is, as a rule, measured by injection of the hydrocarbon which is both the least polar and the least sorbed mobile phase component. However, from the point of detection, this hydrocarbon is often substituted with its close homologue in dead volume measurement.

The second streaming current response, registered in mobile phases of suitable composition, is caused by the vacant zone of the polar component of the mobile phase. This is the very component that is preferentially sorbed on silica gel surface if this is wetted with the mobile phase. Therefore, the elution volume of the polar component of the mobile phase cannot precisely represent the column dead volume. In order to be able to evaluate the mobile phase volume in which the vacant zone is eluted, its elution volume was compared with the dead volume of the bed and with the total liquid phase volume in the bed. Regarding the fact that, for a good generation of the streaming current, addition of alcohol to the mobile phase is required, we selected a mobile phase with such an alcohol amount that a complete, pre-adsorbed alcohol monolayer [22,23] could be assumed.

The total volume of the liquid phase in the column, V_Σ, was found from the weight loss after drying the bed wetted with 95:5 hexane-butanol, mobile phase [24]. In accord with the common practice and ref. [21], the elution volume of n-octane, measured by refractometric detection, was considered as the dead volume of the column (V_M). The influence of the molecular weight of the used hydrocarbon on the measured column dead volume was tested by measuring the elution volume of squalane. The elution volumes of n-octane and

squalane were compared in two systems: in one where, according to the existing views [22,23], the pre-adsorbed monolayer of the polar component should be complete (mobile phase: 99.3:0.7 hexane-ethanol) and in another where this monolayer should be incomplete (hexane containing 0.05 % ethanol). As a practically suitable alternative to refractometric detection, we checked the possibility of calculating the elution volumes of octane and squalane from the wave-like form of the baseline of the photometric detector whose response is affected by the refractive index of the mobile phase. The changes in the baseline can be evaluated in three different ways (Fig. 2).

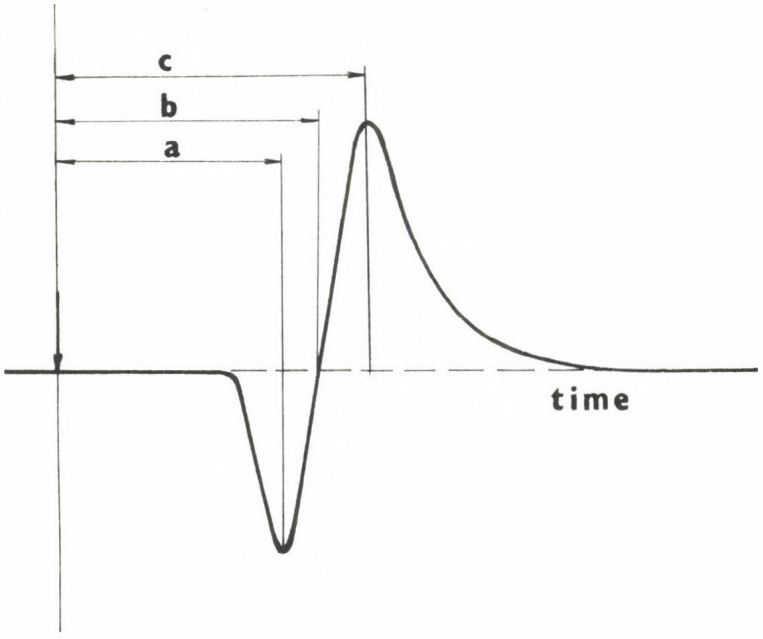

Fig. 2
Procedures for the calculation of the elution volume of hydrocarbon from the fluctuation of the photometer's baseline

a - calculated from the first extreme
b - calculated from the intercept of the recording with the baseline
c - calculated from the second extreme

Procedure b is usually recommended in literature (see e.g., ref. [25]). However, it follows from the results summarized in Tables 2 and 3 that, in the majority of the studied chromatographic systems, refractometric data on the elution volumes range between the values of the elution volumes calculated from the first extreme of the response (Procedure a) and from the intercept of the wave with the baseline, caused by the injection of a saturated hydrocarbon (Procedure b). However, without comparing the results obtained from the baseline of the photometric detector with refractometric measurements it is impossible to decide which variant of the evaluation provides the value closest to the column dead volume.

Elution volumes of squalane are systematically lower than the elution volumes of n-octane. The magnitude of the deviation, which, in the studied systems, was 2.4-9.5 % of the elution volume of n-octane, depends on the used stationary phase and on the composition of the mobile phase. Differences in the elution volumes of n-octane and squalane can only be explained by the partial exclusion of the larger squalane molecule. Its mass is almost four times greater (422.8 dalton) than that of n-octane (114.4 dalton) [26]. With the used column dimensions, the volume of the liquid phase inside the particles, which is equal to the difference (V_{Σ} - V_E), is generally about 2 ml (Table 4). Therefore about 10-25 % of the pore volume and thus, considering the pore diameter distribution, at least 10-25 % of the internal surface of the studied stationary phases are not accessible to the squalane molecules. Obviously the use of close hydrocarbon isomers, differing only by two or three carbon atoms, cannot affect the measured column dead volume. Elution volumes of vacant streaming current responses will never be outside the range of the elution volumes limited by the values of V_M and V_{Σ} (Table 4). Since the values of V_M and V_{Σ} differ by 10 % at the maximum, the deviation of V_V from any of these values will also be within this tolerance.

Table 2. Elution volumes of n-octane and squalane calculated from refractometric and photometric detection. Mobile phase: hexane with the addition of 0.7 % ethanol

Sorbent	n-Octane				Squalane				ΔV [ii] (%)
	Detection				Detection				
	Refractometric	Photometric			Refractometric	Photometric			
		a [i]	b [i]	c [i]		a	b	c	
Lichrosorb Si60	4.11	3.98	4.17	4.50	3.80	3.62	3.75	4.21	-7.54
Silasorb	3.96	3.72	3.78	3.98	3.64	3.47	3.56	3.63	-8.09
Q 15 -10 AW	4.15	4.08	4.17	4.30	3.91	3.85	3.94	4.11	-5.80
Q 15 - 6 AW	4.09	4.17	4.30	3.85	3.85	3.85	3.94	4.14	-5.80
Q 15 - 2AW	4.15	4.14	4.28	4.47	3.94	3.85	4.04	4.24	-5.08
P 129 - 3AW	4.24	4.17	4.28	4.41	4.03	3.95	4.05	4.21	-4.83
P 129 - 5AW	4.13	3.94	4.01	4.24	3.74	3.72	3.88	4.01	-9.45
P 129 - 7AW	4.11	-	-	-	3.90	-	-	-	-
P 129 - 8AW	3.77	-	-	-	3.53	-	-	-	-

i) Procedure for the evaluation of photometric detection (see Fig. 2);

ii) ΔV represents the difference in the elution volume of squalane measured by refractometric detection, relative to the elution time of n-octane.

Table 3. Elution volumes of n-octane and squalane calculated at the refractometric and photometric detection. Mobile phase: hexane with the addition of 0.05 % ethanol

Sorbent	n-Octane				Squalane				$\Delta V^{ii)}$ (%)
	Detection				Detection				
	Refracto-metric	Photometric			Refracto-metric	Photometric			
		a[i)]	b[i)]	c[i)]		a	b	c	
LiChrosorb Si60	4.26	4.04	4.24	4.30	4.02	3.75	3.91	4.04	-5.67
Silasorb	4.11	3.99	4.13	4.23	3.78	3.64	3.76	3.84	-8.03
Q 15 - 10AW	-	4.27	4.37	4.43	-	4.01	4.15	4.24	-
Q 15 - 6AW	4.17	4.11	4.24	4.30	4.07	3.96	4.08	4.23	-2.41
Q 15 - 2AW	4.28	4.19	4.35	4.53	4.08	3.91	4.12	4.23	-4.69
P129 - 3AW	4.24	4.21	4.28	4.37	4.06	3.99	4.11	4.30	-4.26
P129 - 5AW	-	4.14	4.24	4.40	-	3.86	3.99	4.24	-
P129 - 7AW	4.22	4.26	4.35	4.56	3.98	4.11	4.13	4.27	-5.64
P129 - 8AW	3.81	3.89	4.02	4.08	3.63	3.67	3.81	3.91	-4.74

i) Procedure for the evaluation of photometric detection (see Fig. 2);

ii) ΔV represents the difference in the elution volume of squalane measured by refractometric detection relative to the elution time of n-octane.

Table 4. Characteristics of the beds

Sorbent	V_E (ml)	V_M (ml)	V_V (ml)	V_Σ (ml)	ε_E	ε_I	ε_V	ε_Σ	G (g)	$V_\Sigma - V_E$ (ml)
Nucleosil	2.11	-	4.26	-	0.374	0.380	0.754	-	3.16	-
Hypersil	2.19	-	4.30	4.38	0.388	0.373	0.761	0.775	3.22	2.19
Separon	2.07	4.81	4.95	4.94	0.366	0.510	0.876	0.874	1.97	2.87
LiChrospher	2.13	4.10	4.51	-	0.377	0.421	0.798	-	2.03	-
LiChrosorb	2.33	4.11	4.21	4.32	0.412	0.324	0.736	0.765	2.74	1.99
Silasorb	2.26	3.96	3.96	4.25	0.400	0.301	0.701	0.752	3.02	1.99
Q 15 - 2AW	2.73	4.15	4.23	4.53	0.483	0.266	0.749	0.801	2.79	1.80
Q 15 - 6AW	2.68	4.09	4.25	4.59	0.474	0.282	0.752	0.812	2.72	1.91
Q 15 - 10AW	2.67	4.15	4.30	4.62	0.473	0.288	0.761	0.818	2.70	1.95
P 129 - 3AW	2.65	4.24	4.32	4.52	0.469	0.296	0.765	0.800	2.69	1.87
P 129 - 5AW	2.79	4.13	4.29	4.59	0.493	0.266	0.759	0.812	2.70	1.80
P 129 - 7AW	2.67	4.11	4.25	4.68	0.473	0.279	0.752	0.828	2.68	2.01
P 129 - 8AW	2.72	3.81	3.98	4.40	0.481	0.223	0.704	0.779	2.59	1.68

If the elution volume of the first streaming current response is identical to the exclusion volume of the column, it must be (with the same bed geometry) reproducible for columns packed equally with sorbents, having particles of the same shape. It must not depend on the internal structure of the particles in the bed either.

It is obvious from Table 4 that the exclusion volumes of the columns reproduce very well when the columns were packed by the used technique, with hydrothermally modified irregular silica gels of the Silasorb type, having the mean particle diameter of 15 /um. Generally, the measured exclusion volumes differ from the mean value of 2.70 ml by less than 1 %; only in one sample was the deviation 3 %. The specific pore volume was practically constant as well as the weight of the sorbent in the column. This proves the very good reproducibility of the packing procedure which is also obvious from the high efficiencies of the columns (see Experimental). With spherical silica gels, having a mean particle diameter of 10 /um (Nucleosil, Hypersil, LiChrospher), the measured values of V_ε differ by 1.5 % or less from the mean value of 2.10 ml. The disagreement in the weight of these sorbents in the column should be ascribed to the significant differences in their specific pore volumes. The difference in the exclusion volumes of LiChrosorb and/or Silasorb and coarser hydrothermally treated Silasorbs can easily be explained by a particle size distribution. The same holds for Hypersil and the other spherical silica gels.

Use of Streaming Current Responses

As shown already above, the elution volume in which the first streaming current response is registered can be considered identical to the exclusion volume, with a relative error of several percents at the maximum. The vacant response can be used for an approximate determination of the column dead volume. The values obtained in this way will be somewhat higher than the real dead volumes. It can also be used for the determination of the total volume of the liquid phase

in the column; here somewhat lower values are obtained. With both approximations, the relative error can be up to 10 %.

If the volume of the empty column tube (bed dimensions), V_o, is known, the external bed porosity ε_E can be calculated from the elution volume of the exclusion streaming current response according to the following relationship:

$$\varepsilon_E = V_E/V_o \qquad (1)$$

For the determination of the internal bed porosity, ε_I, and of the total bed porosity, ε_T, the total volume of the liquid phase in the bed has to be known. Its determination by drying [22] is time-consuming. Moreover, this operation can have an adverse affect on the column efficiency. For a rapid and easy determination of ε_I and ε_T it is therefore expedient to consider $V_\Sigma \doteq V_V$. The pertaining relationships have then the forms

$$\varepsilon_I = (V_V - V_E)/V_o \qquad (2)$$

$$\varepsilon_T = V_V/V_o \qquad (3)$$

The error in the determination of the total bed porosity, ε_T, caused by the approximation $V_\Sigma \doteq V_V$, is illustrated in Table 4. We use the symbol ε_V for the values calculated from the elution volumes of the vacant streaming current response, V_V, and the symbol ε_Σ for the values calculated from the volumes of the liquid phase found by drying.

If the weight of the sorbent in the bed, G, is known, the specific pore volume, V_p, can be calculated from the volume of the liquid phase in the pores. The latter is equal to the difference $(V_\Sigma - V_E)$:

$$V_p = \frac{1}{G} (V_\Sigma - V_E) \qquad (4)$$

Using the approximation $V_\Sigma \doteq V_V$, we can write that

$$V_p = \frac{1}{G} (V_V - V_E) \qquad (5)$$

The precision of the specific pore volume determination by titration with water is ± 20 % [12,13]. In the calculation of V_p according to Eq. (5), the error will never be greater.

It is obvious that

$$V_o = V_E + G \cdot V_p + G/\varrho \qquad (6)$$

where ϱ is the specific weight of the silica gel. The specific pore volume can also be determined from this relationship arranged in the following form:

$$V_p = \frac{1}{G} (V_o - V_E - G/\varrho) \qquad (7)$$

It can be seen from Table 1 that except for Nucleosil the specific pore volumes calculated from Eqs. (5) and (7) agree with the values obtained by titration with water. V_E and G were taken from Table 4 and the specific weight of translucent fused silica, 2.07 g/ml, [27] was used as the specific weight of all silica gels investigated. V_o was measured as 5.65 ml.

Using Eq. (5), we can rearrange Eq. (6) expressing the volume balance in the following form:

$$\varrho = \frac{G}{V_o - V_V} \qquad (8)$$

From this relationship we can calculate the specific weight of the silica gel skeleton even without knowing the specific pore volumes.

Table 5 summarizes the specific weights of silica gel calculated in this way. This table demonstrates that the specific weight of translucent fused silica is an acceptable first approximation for the density of the skeleton, both for spherical and irregular silica gels.

Using the specific weight of Nucleosil, calculated according to Eq. (8) (ϱ = 2.27 g/ml), we can write the balance of the volume in its bed according to Eq. (6). The respective measured values for V_E and G were 2.11 ml and 3.16 g. If we use the specific pore volume obtained by

titration with water (V_p = 1.0 ml/g), then the calculated value of the bed volume is V_o = 6.66 ml. On the other hand, using the specific pore volume calculated according to Eq. (5) (V_p = 0.68 ml/g), we obtain V_o = 5.66 ml. The bed volume V_o determined from column dimensions is 5.65 ml. If the value of 2.07 g/ml (the specific weight of translucent fused silica) is used as the specific weight of Nucleosil, then calculation according to Eq. (6) will result in the respective V_o values of 6.80 or 5.80 ml. The specific pore volume of Nucleosil, obtained from the elution volumes of streaming current responses, thus appears more realistic.

Table 5. Densities of silica gels calculated from Eq. (8)

Sorbent	ρ (g/ml)
Nucleosil	2.27
Hypersil	2.38
Separon	2.18
LiChrospher	1.79
LiChrosorb	1.83
Silasorb	1.80
Q 15 - 2AW	1.95
Q 15 - 6AW	2.00
P 129 - 3AW	2.02
P 129 - 5AW	1.98
P 129 - 7AW	1.91
P 129 - 8AW	1.55

For the calculation of the mean pore diameter D, the following relationship is commonly used for silica gels [12,28,29]:

$$D = 4\ V_p/S \qquad (9)$$

(where S is the specific surface area). This relationship

can be rearranged into the following form using Eq. (5):

$$D = \frac{4}{S \cdot G} (V_V - V_E) \qquad (10)$$

Table 1 compares the values calculated according to Eqs. (9) and (10) from the specific pore volumes measured by titration with water and from the elution volumes of the streaming current responses. In these calculations the values measured by thermal nitrogen desorption [10] were taken for S.

CONCLUSIONS

From the elution volumes of exclusion and vacant streaming current responses we can obtain a complete description of the liquid phase distribution in the bed. The volume of the liquid in the interparticle space (the exclusion bed volume), the volume of the liquid in the pores of the packing and the total volume of the liquid in the bed can be determined in this way. Considering the total bed volume (bed dimensions), we can express these data by the respective porosities. Knowing the amount of silica gel in the bed, the specific weight of the silica gel skeleton can be easily calculated from the total bed volume and from the elution volume of the vacant streaming current response. Provided that we also know the specific surface area of the solid phase, we can calculate its mean pore diameter. Moreover, we can determine the real linear velocity of the mobile phase in the bed [9] from the elution time of the exclusion streaming current response and from the column length.

In mobile phases of suitable composition both streaming current responses can be generated by the injection of any retained solute or the polar component of the liquid phase. The time required for the measurement is practically the same as the time required for the determination of the column dead volume. Thus the measurement of the elution volumes of these streaming current responses is very rapid and easy. The measurement can be considered as rapid even if the time required for bed preparation is included into

the total time required for the experiment. It should also be emphasized that several data can be obtained on the bed and the sorbent from a single measurement. If the amount of the liquid phase in the pores, the specific pore volumes of the packing, its mean pore diameter or the specific weight of the silica gel skeleton are to be determined, the quality of the column packing is not important.

In many commonly used normal phase chromatographic systems, both streaming current responses are generated at every measurement. Therefore, their elution volumes represent additional information obtained automatically, which can be utilized if necessary. This fact provides a new possibility for the study of these characteristics of the silica gel bed and for the evaluation of chromatographic silica gels and their beds.

The phenomenon responsible for the generation of the streaming current in the chromatographic bed, charge on the solid surface in contact with a liquid of suitable composition, is general. Therefore, the described possibilities of studying particulate material beds should be also applicable to any type of solid particles, regardless whether they are used for chromatographic purposes (e.g., sorbents, catalysts, fillers etc.). This assumption is supported by the possibility of sensing streaming current on the surfaces of qualitatively quite different solid phases [1-4,6,8,9] .

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STUDY OF SOLUTE-SOLVENT INTERACTIONS BY GEL PERMEATION CHROMATOGRAPHY

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SUMMARY

The behaviour of polyurethane prepolymer (PP), based on 4,4'-diphenylmethanediisocyanate (MDI), and polyethylene glycol adipate, in two solvents has been investigated by gel-permeation chromatography (GPC). Tetrahydrofuran (THF), and dimethylformamide (DMF), were used as the solvents. Different chromatographic systems have been used such as: PP/THF/Poragel, PP/DMF/Poragel, PP/THF/porous glass and PP/DMF/porous glass. The shapes and shifts of the peaks show changes in the solution structure of PP. They are considered in respect to intra- and intermolecular interactions of the PP molecules, to kinetic and thermodynamic dissolution properties of the solvents and to preferential interactions among solute, solvent and gel.

INTRODUCTION

Size-exclusion chromatography, classically known as gel-permeation chromatography, is one of the major types of liquid chromatography. It is of growing importance in problems related to characterization of synthetic polymers, particularly according to molecular mass and molecular mass distribution. These values can be obtained from the gel- chromatographic patterns by appropriate calibration of the GPC system and by the interpretation of the chromatograms. There are low GPC systems where

the size exclusion mechanism, controlled by size and shape of polymer in solution is the only mechanism of separation.

For such systems a so-called universal calibration plot, log M vs. elution volume plot, can be established (1). In the systems where solutes have preferential affinity for the mobile phase, the stationary phase, or the gel, a second separation mechanism is present (2-6), the universal calibration is not valid (7-8) and the shape of the chromatogram is not influenced only by the hydrodynamic volume of the solute. Therefore, for precise GPC data analysis of an unknown solute, it is important to study the solubility of the sample in the GPC solvents and the behaviour of the sample in different GPC systems.

A number of investigators (9) have studied the properties of polyurethanes but little work has been reported concerning the gel chromatographic investigation of polyurethane prepolymers (PP).

The purpose of this work is to study the solubility of PP in GPC eluents and to examine the applicability of GPC characterization on the same PP.

EXPERIMENTAL

Materials

The measurements were performed on the solution of the polyurethane prepolymer based on 4,4'-diphenylmethanediisocyanate and polyethylene glycol adipate. Analytical-grade tetrahydrofuran (THF) and dimethylformamide (DMF) were used. The solvents were separately treated before use by the procedure developed in our laboratory. A series of well- defined polystyrene standards (Waters Associates, Milford, MA, USA, ArRo Labs., Joliet, IL, USA) were used for testing the GPC columns.

Procedures

A Waters Associates GPC system consisting of the Model 6000A solvent delivery system, Model U6K universal injector and

R 401 differential refractometer were used in our investigations.

Controlled porosity glasses (CPG), manufactured by Corning (Corning, NY, USA) with pore sizes of 40Å and 100Å, and Waters Poragel columns with the nominal exclusion limits of 60Å and 100Å were used as the solumn packings.

The flow rate was 1 cm^3/min and 0.5-1 cm^3 volumes were injected. The concentration of the samples was 2 mg/cm^3.

RESULTS AND DISCUSSION

In the first part of our work the solubility of PP in THF and DMF was studied. Both solvents dissolve PP but the speed of dissolution of PP in THF is surprisingly high. Solutions were observed at controlled conditions by frequent visual inspection. The solution of PP in THF was less stable. It became first turbid and later the solution separated into two phases. At an elevated temperature the cloud point and separation appeared earlier. The solution of PP in DMF was stable under the same conditions. Only dry THF may be considered as a kinetically good solvent for PP (10); dry DMF is thermodynamically good as well.

The specific behaviour of PP in solution is mainly connected to the components used in the synthesis of PP as well as to characteristics of the solvents used (hygroscopicity). The existence of chemically different bonds in the PP chain enables the formation of intra- and intermolecular physical and hydrogen bonds. The strong intermolecular interaction of different nature in PP solution could lead to phase separation.

Because THF has been shown to absorb moisture from the air if stored in unsufficiently closed vessels (11), physical and chemical reaction between PP and water is also possible.

In further investigations we tried to use gel- chromatographic separation of the PP solution in order to elucidate the behaviour of the PP solution. A series of separations were performed in the chromatographic system; the experimental results are summarized in Figs 1-4.

Refractive index difference, Δn, vs. the elution volume, V_R, is plotted for all the systems studied. The injection of each sample was repeated at least twice and the reproducibility was satisfactory.

The elution behaviour of the PP/THF/Poragel system is shown in Fig. 1.

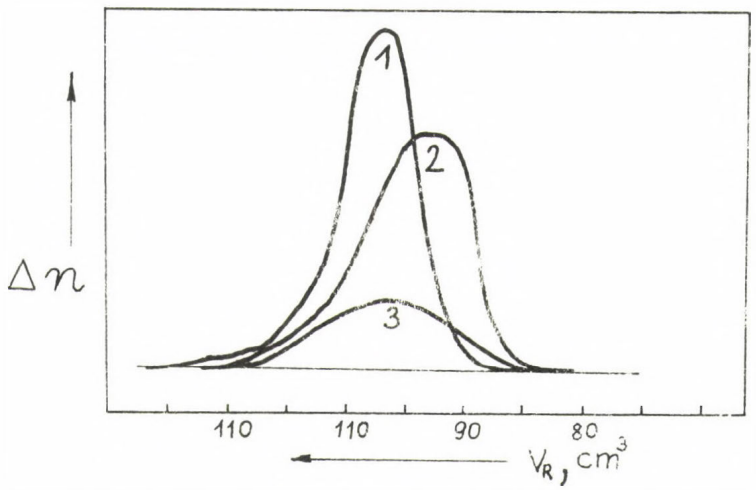

Fig. 1. Chromatograms obtained in the GPC system PP/THF/ Poragel 60 Å, 100 Å, curve. 1 fresh solution, 2 solution aged at controlled condition, 3 sol phase after phase separation

The curve corresponding to the aged PP solution (curve 2) is located at lower retention volume range than the curve for the fresh solution curve (curve 1). Aggregation between the PP molecules could result in such data. Aggregates can be, in principle, broken by heating and carrying out the GPC analysis when the solution has cooled to the operating temperature of the instrument (12). However, the solution of PP (curve 2) reached the cloud point by heating and separated into two phases. Curve 3 represents the chromatogram of the sol phase above the precipitated gel phase. A shift of curve 3 toward higher elution volume is observed. The gel phase did not become soluble in any solvent, no matter what temperature it was

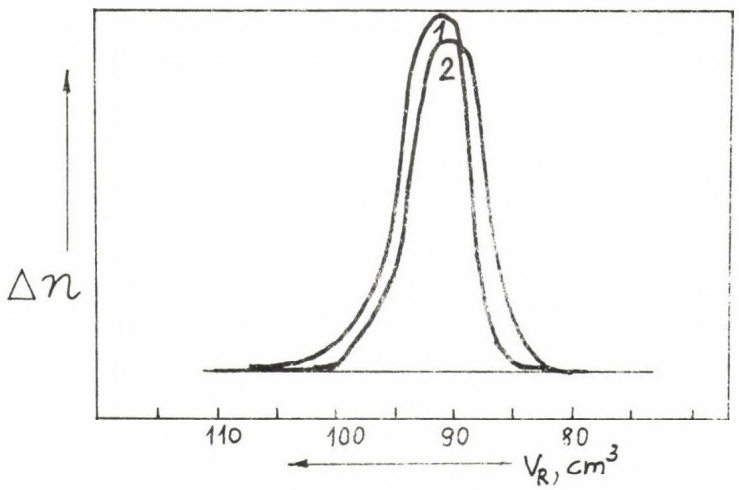

Fig. 2. Chromatograms obtained in the GPC system PP/DMF/ Poragel 60 Å, 100 Å, 1 fresh solution, 2 solution aged at controlled condition

heated to. The separation of the phases occurs in this particular system by simple storage only, regardless of heating.

To make a polymer insoluble it is sometimes sufficient to create at least one bond between each two chains. Therefore, one can conclude that chemical crosslinks occurred with moisture from the air.

The next solvent used as GPC eluent was DMF. Fig. 2. shows the chromatograms obtained in the PP/DMF/Poragel system. A displacement of the chromatogram (curve 2) toward the lower elution volume is observed again. However, the difference in peak elution volume between curves 1 and 2 is small. The aged PP solution became slightly turbid by heating and remained turbid for a long time.

The experiments described above were carried out on the Poragel columns and in solvents of different nature. The results are consistent with the data on solubility parameters of the solvents (3). The highly polar DMF prefers to interact with the most polar component in these systems, with PP. The solution of PP in DMF is, therefore, more stable than the solution of PP in

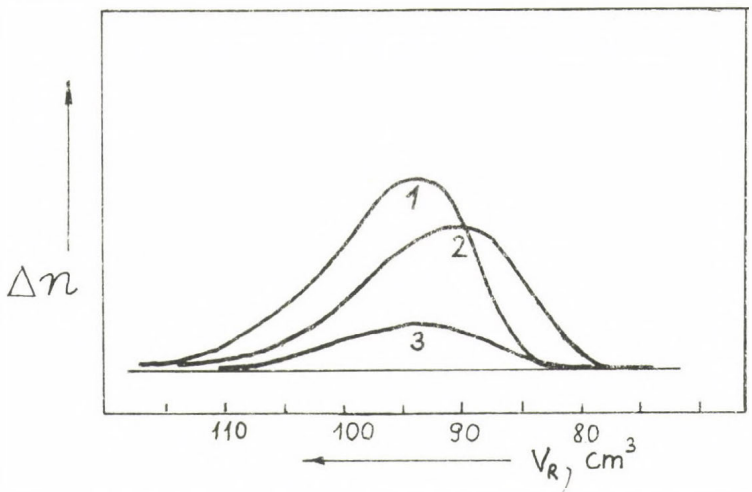

Fig. 3. Chromatograms obtained in the GPC system PP/THF/CPG 40 Å, 100 Å. 1 fresh colution, 2 solution aged at controlled condition, 3 sol phase after phase separation

THF. In addition, the peaks in all the chromatograms obtained on Poragel are nearly symmetrical, which indicates no retardation by PP-gel interactions. Furthermore, the peaks in Fig. 2. are narrower in distribution than the peaks in Fig. 1. and they occur at lower elution volumes than in Fig. 1., in spite of the fact that DMF is a better solvent for PP than THF. Because the PP molecules are eluted earlier than expected it seems reasonable to assume the existence of an additional separation mechanism: the PP molecules seem to be "repulsed" from the gel.

The elution behaviour of PP on CPG packing is illustrated in Figs 3-4. Curves 1 and 2 in Fig. 3. are asymmetric. A tailing in the low molecular mass region may result from PP-CPG interaction effects and cannot be explained only by steric exclusion mechanism. CPG is a highly polar gel with a number of active sites on the surface and it prefers to interact more with the polar PP than with the less polar THF.

The peak of the aged PP solution is again shifted toward lower elution volumes. Curve 3 in Fig. 3 shows the peak in the of uncrosslinked sol phase of the PP sample.

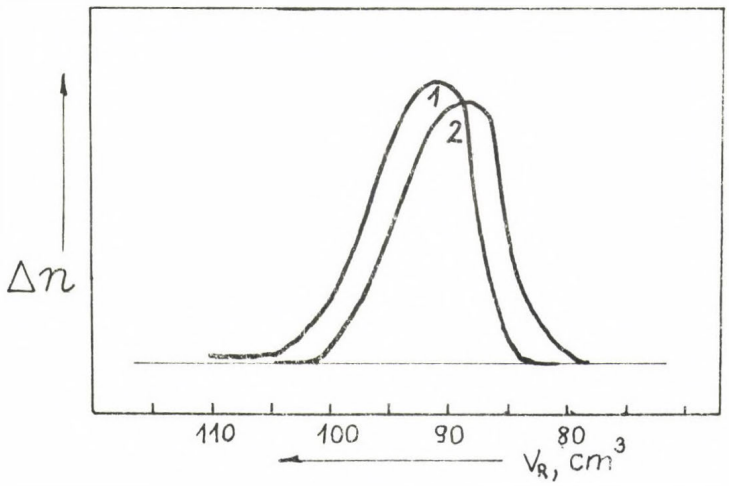

Fig. 4. Chromatograms obtained in the GPC system PP/DMF/ CPG 40 Å, 100 Å, 1 fresh solution, 2 solution aged at controlled condition

The elution behaviour of PP in DMF solution on CPG is shown in Fig. 4.

The results obtained are consistent with the earlier discussion.

CONCLUSIONS

Solubility properties of PP in two commonly used chromatographic solvents, as well as the GPC elution behaviour of PP in the same solvents have been examined.

PP is soluble in dry THF and dry DMF.

Similar effects, somewhat different in magnitude, are observed in both solvents.

A dilute PP solution changes its structure by ageing. A PP solution in THF is less stable. Cloud point and phase separation occurs.

DMF is thermodynamically better solvent than THF. Moisture from the air absorbed by dry THF seems to be the source of this phenomenon. Consequently, erroneous GPC data can result. Shift

of the peak toward lower elution volumes indicates the strong intermolecular interactions.

Inconvenient experimental conditions make the GPC characterizations of PP limited.

Finally, it can be concluded that the most convenient system for gel chromatographic separation of PP, among the considered systems, is PP/DMF/CPG.

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TEMPERATURE DEPENDENCE OF THE RETENTION TIME AND RESOLUTION IN NORMAL-PHASE AND REVERSED-PHASE CHROMATOGRAPHY

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It is well known that in chromatography, the retention time (t_R) decreases as the temperature is increased; most often the relative retention (α) also decreases, ($\alpha = t_{R2}/t_{R1}$, where indices 1 and 2 refer to the first and second components. Usually the relative retention is the ratio of adjusted retention time, but in this work we used unadjusted retention time. It does not influence qualitative results). However, in some cases, abnormal temperature dependences were reported for liquid chromatography, i.e., t_R increased with increasing temperature [1-3]. In the present work we studied this in more detail trying to establish its reasons.

MATERIALS AND METHODS

Adsorption chromatography experiments were carried out on a VKS-12 liquid chromatograph designed in the Special Construction Bureau of the Estonian Academy of Sciences. Columns (6 x 150 mm) were dry-packed with Silosorb 600-30 μm particles. The mixture to be separated which contained equal quantities of nitrobenzene, benzonitrile and benzaldehyde was eluted with a mixture of heptane-dichloromethane-methanol at a ratio of 700:299.75:0.25, except for experiments with varying methanol content. Detection was performed with an UV-detector (cell volume: 7 μl, wavelength: 229 nm).

Reversed-phase chromatography experiments were carried out on a Du Pont 850 liquid chromatograph using a 25 cm x

4.6 mm column packed with Zobrax ODS. Phenacyl ethers of fatty acids were separated using methanol-water (90:10 v/v) as the eluent.

The flow rate of the mobile phase was 1.8 cm^3/min, and the maximal inlet pressure was 250 psi. Detection was also carried out with an UV-detector at a wavelength of 254 nm. In addition to the chromatographic experiments, we also determined the isotherms of methanol sorption from the mixture of heptane-dichlormethane using a statical approach: the Silasorb sample (m = 0.2 g) was placed into hermetically sealed vessels to which solutions were added (volume, V equal 5 ml) with varying initial concentrations of methanol (C_o from 0.04 to 1.2%). The mixture was thermostated for 2 hr on a shaker, a sample solution was taken with a microsyringe, and the equilibrium concentration (C*) determined on a gas chromatograph. The concentration of the matter in the adsorbed state (a*) was calculated from the following equation:

$$a^* = \frac{(C_o - C^*)\ V}{m} \qquad (1)$$

In the same manner, given the initial methanol concentration of 0.028 ml/ml, solution volume 10 ml and Silasorb sample 0.1 g, we measured the kinetics of methanol absorption by a sorbent, taking solution samples at definite intervals and analyzing them on the gas chromatograph.

The analyses were performed on a Tsvet-100 gas chromatograph with a flame ionization detector using 2-m columns packed with the porous polymer sorbents of Polysorb-1 or Porapak P at 130°C. The quantitative analysis of methanol was carried out by the absolute calibration technique.

Water adsorption on Silasorb C_{18} was also investigated. Since it was difficult to reveal minor changes of the water content in the methanol-water mixture at a ratio of 90:10, we studied water adsorption using humidified methylene chloride: after addition of water to methylene chloride in a separating funnel and thorough shaking, we separated the organic layer.

From here on the procedure was similar to the measurement of methanol adsorption kinetics, only difference being that the solution volume was 10 ml and 5 g of Silosorb C_{18} was used. Gas chromatography was carried out at 80° on a column packed with Polysorb-1 and katharometer detector was used.

RESULTS

Working with a column packed with Silosorb 600, we changed the temperature from 22.5 to 40°C. As shown in Table I, this increased the retention time (t_R) and the relative retention (α). The resolution (R) of the component also increased.

Table I.
Retention data at various temperatures on Silosorb 600.
Eluent: 700:299.75:0.25 heptane-dichloromethane-methanol*.

T,°C	22.5	30	40
t_{R1}, s	208	206	217
t_{R2}, s	410	404	440
t_{R3}, s	555	546	613
$\alpha_{2,1}$	1.97	1.96	2.03
$\alpha_{3,2}$	1.35	1.35	1.39
$R_{2,1}$	0.87	0.89	0.97
$R_{3,2}$	1.7	1.77	1.87

* 1 - nitrobenzene, 2 - benzonitrite, 3 - benzaldehyde.

Unfortunately, due to the high volatility of dichloromethane (boiling point: 44°C) it was impossible to carry out the experiments at higher temperatures. The above data were obtained at 0.025% methanol concentration in the eluate. When there was no methanol in the eluent the relationships were as usual: t_R and α decreased as the temperature increased. Table II lists the t_R and α values at varying methanol concentrations and two extreme temperatures, 22 and 40°C. It is of interest that at temperature variations within this range, the produc-

tivity of preparative-scale chromatography also increased [4]. (The productivity P is the sample volume (V) in time unit at given resolution R: $P=V/t_p$, where V - sample volume at which given R is observed, t_p - time of the separation).

Table II.
Dependence of t_R and α on the temperature and the methanol content of the eluent*

% methanol	0		0.01		0.02	
T, °C	22	42	22	42	22	42
t_{R1}, s	277	270	259	265	210	224
t_{R2}, s	607	570	548	559	401	442
t_{R3}, s	925	850	810	815	540	606
$\alpha_{2,1}$	2.19	2.12	2.11	2.11	1.91	1.97
$\alpha_{3,2}$	1.52	1.49	1.48	1.46	1.35	1.37

* Stationary phase: Silosorb 600. Eluent concentration:
0% methanol: 700:300 heptane-dichloromethane
0.01% methanol: 700:299.9:0.1 heptane-dichloromethane-methanol
0.02% methanol: 700:299.8:0.2 heptane-dichloromethane-methanol
1 - nitrobenzene, 2 - benzonitrite, 3 - benzaldehyde.

In reversed-phase chromatographic experiments, the temperature was changed between 35 and 50°C. Table III lists the retention data of phenacyl ethers of fatty acids. On the other hand if pure methanol is used as the mobile phase, the temperature relationship of the retention data is as usual: both t_R and α decrease when the temperature increases.

DISCUSSION

The process occurring in the liquid-absorption system is described by the equilibrium equation [5]:

$$A_m + bM_s \rightleftharpoons A_s + bM_m \qquad (2)$$

where A is the eluted substance, M is the eluate; subscripts m and s refer to molecules in the mobile and stationary phases, respectively. The free energy of the process is described as:

$$\Delta E = E_s^A + bE_m^M - E_m^A - bE_s^M \qquad (3)$$

Table III
Retention data at various temperatures on Zorbax ODS.
Eluent: 90:10 methanol-water

T	^oC	35	40	45	50
$t_{R(12:0)}$	s	13.0	14.6	13.6	12.1
$t_{R(14:0)}$	s	26.1	29.4	27.3	23.1
$t_{R(16:0)}$	s	49.3	57.1	52.7	42.9
$t_{R(18:0)}$	s	93.2	109.1	98.2	78.4
$t_{R(20:0)}$	s	177.2	207.4	186.0	142.8
α(14:0/12:0)		2.00	2.01	2.007	1.909
α(16:0/14:0)		1.889	1.942	1.930	1.857
α(18:0/16:0)		1.890	1.911	1.863	1.827
α(20:0/18:0)		1.901	1.901	1.894	1.821
$t_{R(iso-14:0)}$	s	23.1	23.4	22.6	20.8
$t_{R(iso-15:0)}$	s	32.7	36.8	30.9	27.7
α(iso-15:0/iso-14:0)		1.416	1.573	1.367	1.332
$t_{R(c-18:1/\omega-7)}$	s	53.7	61.7	66.1	47.8
$t_{R(t-18:1/\omega-7)}$	s	58.0	64.9	71.3	51.5
α(t-18:1/ω-7)/(c-18:1/ω-7)		1.08	1.05	1.079	1.077

In experiments on adsorption columns containing Silosorb 600 the methanol concentration in the eluent was very small. Having used the known formula

$$C_{max} = C_o / \sqrt{2\pi n} \qquad (4)$$

where C_{max} is the concentration at peak maximum, C_o is the initial concentration of the eluted substance and n is the

number of theoretical plates, we obtain ($C_o \approx$ 1-2 mg/ml, n = 1000) $C_{max} = 10^{-3}$% under the experimental conditions. Therefore, the methanol concentration in the eluent is only one order of magnitude higher than the maximal concentration of the eluted substance at column outlet. Evidently, under such conditions, one can neglect the methanol - sorbate interaction in the mobile phase; the interaction in the mobile phase can be restricted to interactions with the weakly polar molecules of heptane and dichlormethane. Such interactions are usually much less than adsorption interactions [5] and are hence negligible. Then equation (3) can be reduced to the approximate equality

$$\Delta E \approx E_s^A - bE_s^M \qquad (5)$$

with an increase in temperature, the desorption of methanol from the active sites of the silica gel is more intensive than the decrease in the adsorption of the sorbates:

$$b\,\partial E_s^M/\partial T > \partial E_s^A/\partial T$$

thus even increasing the adsorption of the sorbates:

$$\partial_\Delta E/\partial T = \partial E_s^A/\partial T - \partial E_s^M/\partial T < 0$$

In other words, the silica gel surface, in the presence of methanol in the mobile phase, is substantially blocked by methanol molecules thus impeding the adsorption of the separated substances. At an elevated temperature, methanol is partially desorbed releasing active sites and hence increasing the adsorption. Simultaneously the selectivity of the separation and the resolution also increase with an increase of the retention time.

A significant level of methanol adsorption from its mixture with heptane and dichlormethane is confirmed by the sorption isotherm shown in Fig.1. Methanol adsorption decreases markedly when the temperature is increased from 22 to 35°C.

The assumption that the temperature dependence of the retention time is determined mainly by adsorption phenomena rather than interactions in the mobile phase is also supported by a long period needed for the establishment of equilibrium. Fig.2 shows changes in t_R with time for all three sorbates: from the start of eluent supply to the column (zero point) mixture was injected one after the other and the retention times were measured at each injection. It is seen from this figure that at 24°C, the retention times gradually decrease and become constant only after several hours. At increasing temperature (see the pointers in fig.) t_R drops drastically at first: methanol desorption proceeds slowly and the sorbate responds to the temperature increase by a decrease in the

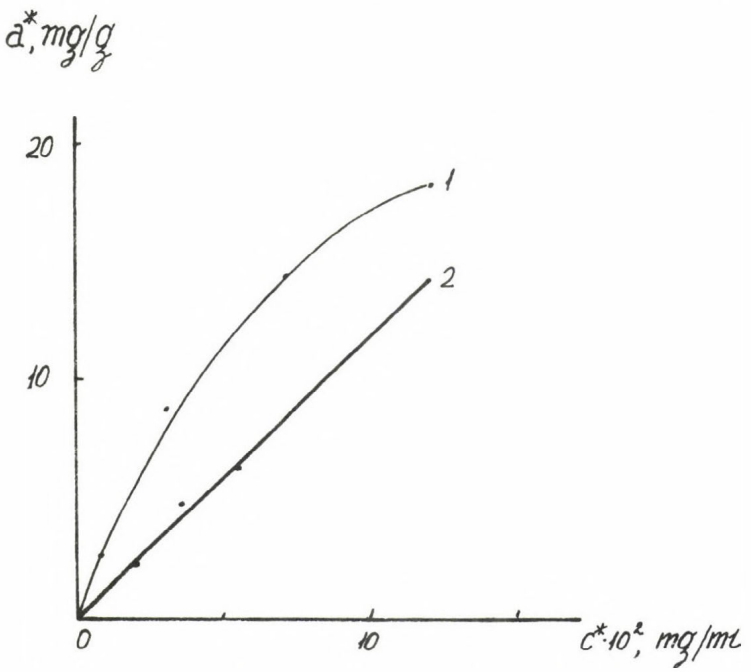

Fig.1. Isotherms of methanol sorption from hexane-dichlormethane mixture of Silosorb 600 at 22° (1) and 35°C (2).

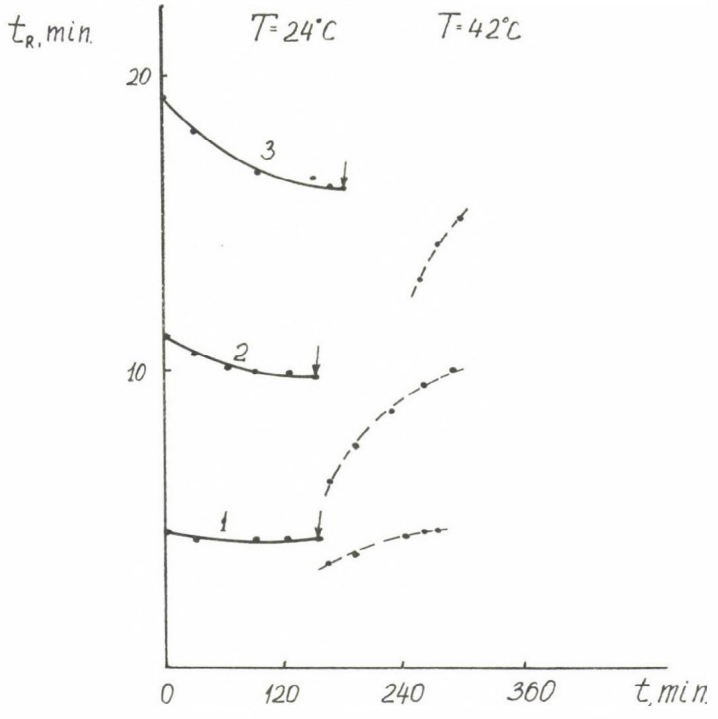

Fig.2. Time dependence of the retention time (t_R) of nitrobenzene (1), benzonitrile (2) and benzaldehyde (3) at 24 (solid line) and 42°C (broken line). The pointers indicate the moment of temperature change from 24 to 42°C.

sorption ability. Gradually methanol is desorbed, the liberated adsorption sites are occupied by the sorbate and its retention time increases. It has been reported [2,3] that it takes about 12 hr to attain equilibrium after a temperature shift. Evidently, the rate of the establishment of a new equilibrium state can indicate whether the process is determined by interactions in the mobile or the stationary phases: at low rate one can be sure that interaction in the adsorbed state plays the main role.

The long period for the establishment of sorption equilibrium with methanol is confirmed by the direct measurement

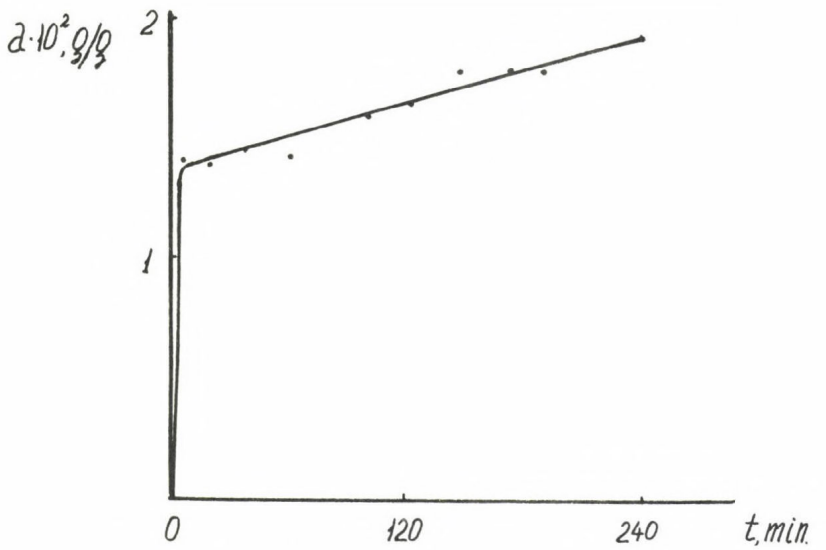

Fig.3. Kinetics of methanol sorption from hexane-dichloromethane mixture on Silosorb 600.

of the sorption kinetics. As seen from Fig.3, though the bulk of methanol is adsorbed within several minutes, this is followed by a slow, several hour long period during which the residual methanol is adsorbed.

It is somewhat more difficult to explain the temperature relationships in the case of reversed-phase chromatography. Evidently, also here, the main processes are those which occur on the adsorbent, since the retention time also changes slowly, similarly as in the normal-phase system.

Since in pure methanol, the temperature relationship is the usual one, one can assume that here, adsorption of water on the free hydroxyl groups preserved after bonding the octadecyl radicals to silica gel plays the main role. The existence of such an adsorption is confirmed by direct measurements of its value on Silosorb C_{18} from humid methylene chloride, which have shown the adsorption of about 25% moisture from the latter. However, this contradicts the repeatedly voiced assum-

ption of the insignificant role of free hydroxyl groups in the adsorption on reversed-phase systems.

In general, we should admit that we failed to completely explain the reasons of the abnormal temperature dependence on reversed-phase systems. This problem needs more comprehensive experimental investigations.

CONCLUSIONS

These studies permitted us to establish that the abnormal temperature dependence of the retention parameters and resolution (their increase with an increase in the temperature) is observed both in normal- and reversed-phase chromatography. It is evidently caused by adsorption of some components of the eluent on the adsorbent which is temperature-dependent. Therefore, temperature should be more widely employed in liquid chromatography as one of the important parameters affecting retention and separation. In pure investigations it is necessary to consider the significant changes in the chemistry of the adsorbent's surface under the action of the eluent.

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THE PRODUCTIVITY OF PREPARATIVE LIQUID CHROMATOGRAPHY WITH LINEAR SORPTION ISOTHERM

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INTRODUCTION

In preparative liquid chromatography, as in gas chromatography, the most important parameter is productivity, that is the amount of the mixture, separated per unit time at a given degree of separation. This parameter is of particular importance when relatively large amounts of substances are separated.

Separation worsens with increasing sample size either due to a large total sample volume (volume overload) or to high substance concentrations in the sample (concentration overload). The effect of volume overload is described in detail by a linear theory while the concentration overload effect should be analysed on the basis of the nonlinear theory of chromatography, which is far from being complete. It is not surprising, therefore, that the relationships connecting productivity with the separation parameters take only into account the volume overload and are applicable only if highly dilute solutions are used.

Such relationships were described in the literature /1, 2/ and were experimentally verified in gas chromatography. Another expression for the productivity has been recently described by Hupe and Lauer /3/ which corroborates the conclusions of the earlier relationships /2/ particularly the influence of the eluent flow rate and the column length. The expression described by Sakodynskii and Volkov /2/ is:

$$P = F \frac{\frac{\alpha+1}{\alpha}}{R+\frac{1}{2}\frac{\alpha-1}{\alpha}} \left(K_s - \frac{1}{2} R\sqrt{\frac{2\pi H_o}{L}}\right), \qquad (1)$$

where F is the eluent flow rate, R is the resolution, $\alpha = t_2/t_1$, $K_s = (\alpha-1)/(\alpha+1)$; t_1, t_2 are the uncorrected retention times of the first and second component, H_o is HETP under no overload, and L is the length of the column.

In this work we studied experimentally how the productivity is affected by such separation parameters as the flow rate and the composition of the eluent, the temperature and length of the column, and the substance concentration in a sample. The experimental results are discussed on basis of eq. 1.

EXPERIMENTAL

A preparative liquid chromatograph VKS-12 with dry-filled columns, 6 mm in diameter, was used. Silasorb 600 (d_p = 30 μm) was taken as the sorbent and heptane-dichloromethane-methanol mixture as the mobile phase. Except in the experiments where the eluent composition was varied, the composition of the eluent was 700:299.75:0.25. The separated mixture contained nitrobenzene, benzonitrile and benzaldehyde in equal amounts. To exclude the influence of concentration overload, the mixture was diluted by excess heptane, so that the concentration used corresponded to the linear region of the sorption isotherm. At least up to concentrations of 1-2 mg/ml the isotherm can be approximated by a straight line. In addition , we studied the peaks widths as a function of the concentration of the compound in heptane. As the benzonitrile and benzaldehyde concentration increases from 0.5 to 2.0 mg/ml, peaks do not broaden, but at a higher conventration they sharply broaden due to concentration overload. For nitrobenzene this broadening is smaller. Up to a concentration of 2 mg/ml, the peaks are rather symmetrical. Therefore, we used a concentration of 1 mg/ml in all experiments except in those where the concentration dependence of productivity was specially investigated.

All calculations were made by standard methods: HETP was calculated from the peak width measured by the tangents at the inflexion points, and the productivity by the method used in gas chromatography, namely, by plotting the resolution R as a function of the sample volume for different values of a given parameter, for example, the flow rate. Furthermore, for a given R, which corresponds to a required degree of separation, one finds a certain sample volume that provides this value of R. Each sample volume is divided by the corresponding separation time, measured from the instant of sample introduction to the disappearence of the tail of the last peak. These ratios are equal to the productivities at different flow rates.

RESULTS AND DISCUSSIONS

Eluent flow rate. As seen in eq. 1, the dependence of the productivity on the eluent flow rate (F) must pass through a maximum provided H_o and F are proportional. Such a dependence was confirmed in gas chromatography /2/. Typical dependencies of P on the flow rate, which we obtained in liquid chromatography, are presented in Fig. 1. In the investigated flow rate range, H_o is proportional to F and, accordingly, the dependencies of the productivity on F pass through a maximum. The general behaviour of this dependence is the same in both gas and liquid chromatography, i.e., the specificity of liquid chromatography does not, play here any role.

Eluent composition. Eluent composition affects the Henry coefficient K of each separated substance and the saparation factor α. The Henry coefficient, according to eq. 1, has no effect on the productivity: as K increases, volume overload decreases and a greater sample volume can be introduced into the column, but the separation time also increases; these two effects compensate each other. On the contrary, even a small variation of α results in a substantial productivity change. The quantities before the bracket in eq. 1 show a comparatively weak dependence on α and the dominant effect on P is produced

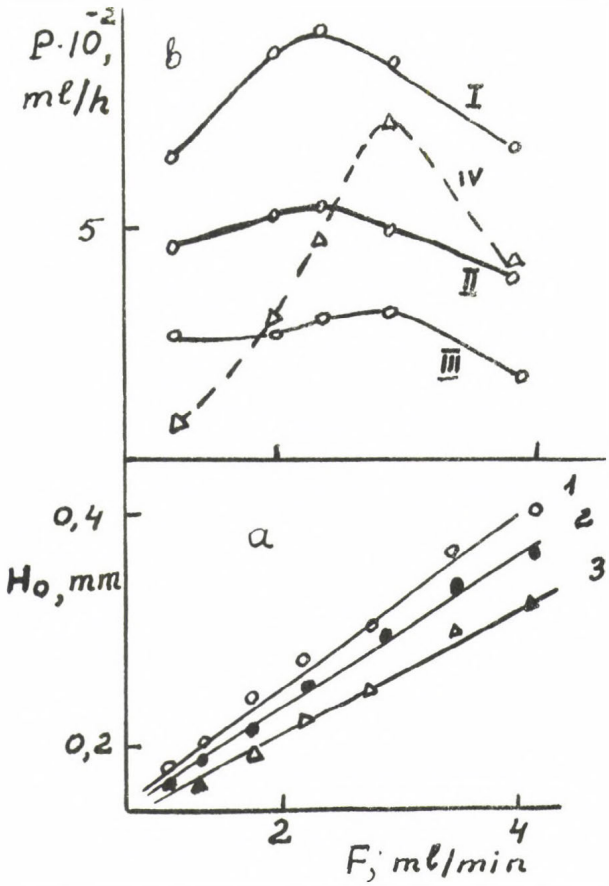

Fig. 1. Dependence of H_o(a) and P(b) on the flow rate (F). 1-nitrobenzene, 2-benzonitrile, 3-benzaldehyde. I-III represent the resolution of nitrobenzene and benzonitrile, R = 1.5; 1.6; 1.7, respectively; IV represents the resolution of benzonitrile and benzaldehyde, R = 0.85

by the selectivity K_s, the dependence between P and K_s being practically proportional.

We also varied the amount of methanol in the eluent. The influence of methanol concentration C on α is shown in Table I.

Fig. 2 presents the experimental plots of productivity versus K_s. As seen they are almost straight lines. Thus, one can change within wide limits the productivity of preparative installations by varying the eluent composition.

Table I. The influence of methanol concentration* on the separation factor (α)**

Methanol concentration in the eluent	α_{21}	α_{32}
0	2.18	1.45
0.01	2.03	1.39
0.02	1.99	1.38
0.025	1.95	1.35

*Proportion of the other components of the eluent:
heptane 700
dichloromethane 299.75

**Subscripts 1, 2 and 3 refer to nitrobenzene, benzonitrile and benzaldehyde, respectively.

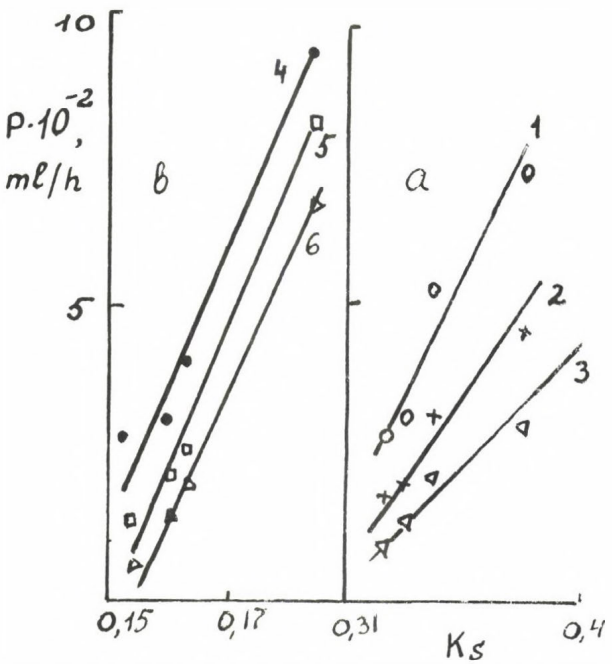

Fig. 2. Depencence of the productivity on the selectivity coefficient for the pair of nitrobenzene-benzonitrile (a), and benzonitrile-benzaldehyde (b): 1-R=1.5; 2-R=1.6; 3-R=1.7; 4-R=0.75; 5-R=0.8; 6-R=0.85

Temperature. The specificity of liquid chromatography concerning the role of the eluent can be observed, at least in some cases, by varying the temperature.

In gas chromatography, as temperature increases, the separation factor α usually decreases, H_o passes through a maximum and, according to eq. 1, the temperature dependence of P also has a maximum /2/. We varied the temperature from 22.5 to 40°C, and the productivity increased. We may, of course, assume that if the temperature is varied in a wider range, a plot with a maximum may occur. However, we could not experimentally verify this fact because the volatility of dichloromethane prevented us from heating the column above 40°C.

The productivity increase is evidently caused by the active role of methanol. The latter is sorbed on Silasorb and thus blocks the active centres of the adsorbent, which, as has already been said, results in a decreased retention time and α. As temperature increases, methanol sorption decreases, which results in an increase in the retention time and α. Such an anomalous temperature behaviour of the retention time was also noted by others /4, 5/. In our experiments the HETP also increased with temperature. An increase in α and a decrease in

Table II. The influence of temperature on the analytical results*

T, °C	22.5	30	40
α_{21}	1.97	1.96	2.03
α_{32}	1.35	1.35	1.39
H_{01}, μm	344	295	255
H_{02}, μm	332	316	295
H_{03}, μm	293	271	282
P_{21}(R=0.65), μl/h	521	727	768
P_{21}(R=0.85), μl/h	122	166	209
P_{32}(R=1.13), μl/h	567	742	768
P_{32}(R=1.5), μl/h	276	409	475
P_{32}(R=1.7), μl/h	92	166	279

*T = temperature, $\alpha = t_2/t_1$, H_o = the HETP under no overload, P = productivity, R = peak resolution. Subscripts 1, 2 and 3 refer to nitrobenzene, benzonitrile and benzaldehyde, respectively

H_o with temperature causes an increase in productivity, as seen from the data compiled in Table II, obtained for 0.025% concentration of methanol in the eluent.

Column length. According to eq. 1, the productivity at first increases with an increase in the column length, but gradually this increase slows down and the productivity approaches an asymptotical value.

We investigated columns with the lengths of 150, 250 and 400 mm. The first two columns consisted of one while the third column consisted of two sections, 150 and 250 mm long, connected together with a capillary passage. The sorbent in the sections was not packed newly, we just used the short columns from the previous experiments. The separated mixtures consisted of nitrobenzene, benzonitrile and benzaldehyde and anisole and nitrobenzene; heptane-dichlormethane was used as the eluent. The dependence of productivity on column length is shown in Fig. 3. The plots are straight lines. For the long column the experiment did not find any inhibition of the productivity increase as predicted by the theory. The productivity growth may be slowing down in columns even longer than ours.

In eq. 1 the whole expression before the bracket does not depend on column length, and it may be regarded merely as a scale factor: the behaviour of the plot is entirely determined by the expression in brackets. We calculated this expression for the experimental values of K_s and H_o. The corresponding theoretical curve for

$$A = K_s - 0.5\ R\ \left(\frac{2\pi H_o}{L}\right)^{1/2}$$

(which is the term in brackets in eq. 1) is also shown in Fig. 3. Its curvature is not large and, in first approximation, the relationship can be represented by straight lines. The qualitative agreement between the theoretical and experimental curves may be considered as satisfactory.

Substance concentration in the sample. In the linear region of the sorption isotherm the concentration of the separated substances in a sample does not affect separation. Hence,

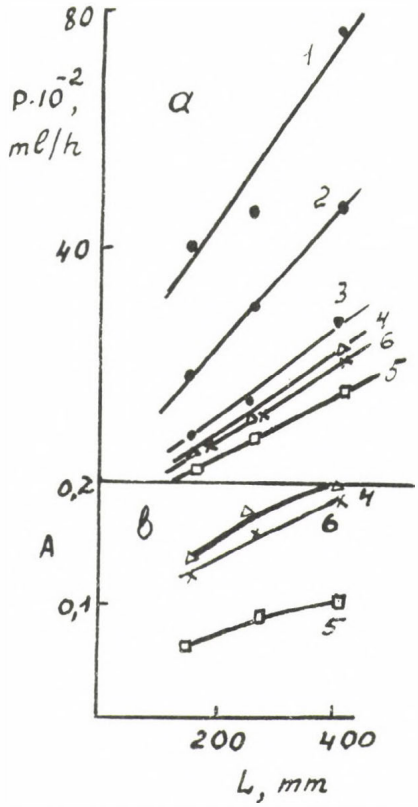

Fig. 3. Dependence of productivity (a) and A (b) on column length: 1-3: anisole-nitrobenzene, R=1.1; 1.2; 1.3; 4-5: nitrobenzene-benzonitrile, R=3.2; 3.5; 6: benzaldehyde-benzonitrile, R=1.65

in order to obtain larger productivity it is reasonable to use more concentrated samples. However, when the concentration is higher than the point up to where the isotherm is linear, the situation changes: as the concentration increases, the peak broadens and, accordingly, the resolution becomes worse; i.e., concentration overload will be observed. Naturally, the question arises whether one should increase concentration and compensate the overload by reducing the sample volume or work at low concentrations. In the literature the second route is recommended /6/, but this is neither well explained, nor confirmed by productivity measurements.

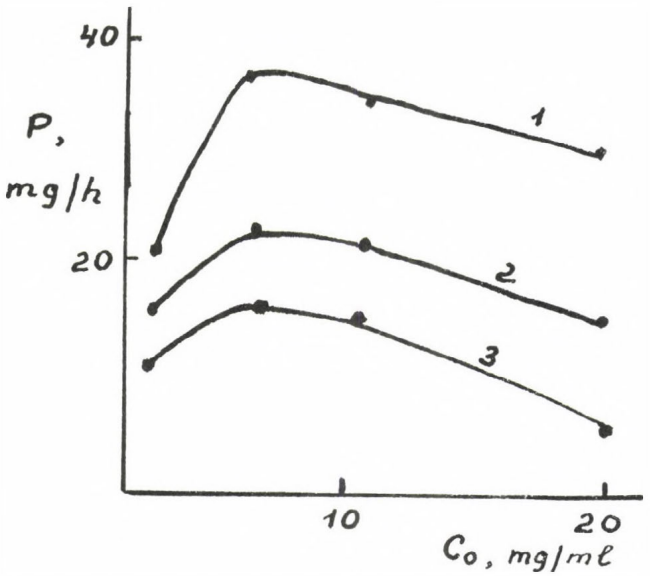

Fig. 4. Dependence of productivity on concentration. Samples: nitrobenzene-benzonitrile. 1:R=1.4; 2:R:1.6; 3: R=1.8.

We have studied the influence of concentration on the productivity by introducing different volumes of a ternary mixture containing 1, 5, 10 and 20 mg/ml of components. Using the results of these experiments, we plotted resolution R against the sample volume, from which we calculated, as usual, the volume V_s that provided a given R. The productivity was calculated by the expression $P = V_s C/t$, where C is the substance concentration in the sample and P is expressed in mg/h.

In calculating R it is convenient to use in the denominator not the actual total peak widths, but the doubled sum of two half-widths facing each other. This is recommended because at high concentrations the isotherm is no longer linear and some, though not large, peak assymmetry can be observed. However, the general pattern of the dependence of productivity on concentration does not markedly change.

The dependence of P on the concentration of the separated substances is given in Fig. 4. As the concentration increases, the productivity passes through a maximum whose position ap-

proximately corresponds to the concentration at which the sorption isotherm starts to deviate from linearity. The ascending branch of the plot confirms the already mentioned rather obvious conclusion that in the linear region it is expedient to use more concentrated solutions, while the decrease after the maximum shows the undesirability of large concentration overloads. In view of this, the studies of separation in the linear sorption isotherm range are not only of theoretical, but also of practical importance.

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THE COMPARISON OF THE PROPERTIES OF C_{18} FILMS BONDED TO SILICA GELS AND CONTROLLED POROSITY GLASSES

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The solids most frequently used as the support for chemically bonded phases are silica gels. This results from their easy availability and relatively low costs of preparation. The presence of the surface hydroxyl groups permits to graft different kinds of radicals to the silica gel surface. The most popular are the aliphatic radicals, e.g. C_2, C_8, and C_{18} chains. The maximal density of the bonded chains on the silica gel surface, using monochlordimethyloctadecylsilane as the bonding agent, reaches 3.5 - 3.8 μmol/m^2. Using aminosilanes for the bonding reaction, even 4.0 - 4.3 μmol/m^2 coverage may be obtained /1/.

The characteristic feature of aliphatic bonded films is the phase transition occurring in the temperature range usually applied in HPLC practice (19 - 25°C for C_{18} chains on silica gel). This phase transition is connected with the configuration change of alkyl chains: below 19°C the alkyl chains form a sort of condensed phase while at a higher temperature the bonded films change into liquid expanded films /2/. According to Serpinet /1/ this phase transition leads to a change of the inclination of the radicals to the support surface. As a result of this phenomenon the retention mechanism of chromatographed substances also changes. In extreme cases a small temperature variation can even cause a change in the peak sequence. The effect described above is of great importance for HPLC because changes in the bonded chain configuration cause a change in the composition of the surface layer which is built of alkyl radicals and mobile phase molecules. The phase transition occurring

in the alkyl films bonded to silica gel takes place even at maximal densities of coverage. In order to avoid a confusion caused by the phase transition, an experiment should be carried out either at a precisely determined temperature or by using a longer or shorter bonded alkyl chain, e.g. C_{22}.

Our previous investigations /3, 4/ have shown that column packings obtained by bonding ODS to controlled porosity glasses (CPG) are very interesting. The properties of C_{18} films bonded to CPG surface are different than those on silica gel. As it was found these differences result from the presence of boron atoms on the glass surface.

EXPERIMENTAL

Controlled porosity glass (CPG) was obtained from Vycor type glass by proper thermal treatment and the leaching procedure described elsewhere /5, 6/. Two particle fractions were prepared: 150 - 200 μm for gas chromatographic measurements and 10 - 12 μm for HPLC investigations. The individual portions of each fraction were heated at 700°C for 5 or 70 hr. The latter operation was performed in order to enrich the glass surface in boron atoms /7, 8/. The mechanism of this process has been discussed in ref. /9/. The surface of thermally treated CPGs was then rehydroxylated.

Dimethyloctadecylmonochlorsilane (ODS) was bonded on the prepared supports according to the procedure described in ref. /4/.

C_{18} chains were also bonded on 5 μm Lichrosorb Si-100 (E. Merck, Darmstadt, FRG). The preparation procedure was the same as for glasses.

"Inverse" gas chromatography measurements were performed using a Chrom 4 gas chromatograph (Laboratorni Pristroje, Czechoslovakia) with a katharometer detector. Glass columns, 0.6 m x 3 mm i.d., were used. 0.02 μl of n-heptane was injected with a 1 μl Hamilton syringe.

A Pye Unicam model LC 20 liquid chromatograph with UV detector (254) was employed.The studied materials were packed into

Table 1. Coverage density of C_{18} chains bonded to silica gel and glasses

Material	Density of coverage, μmol/m²	
	Particle diameter 100-200 μm	Particle diameter 10-12 μm
Lichrosorb Si-100/C_{18}	-	3.12*
Glass O/C_{18}	1.87	1.59
Glass 5/C_{18}	2.44	2.30
Glass 70/C_{18}	5.03	4.55

*Particle diameter 5 μm

100 x 4 mm i.d. stainless columns. Methanol - water mixtures were used as the mobile phases.

Specific surface areas were determined from the thermal desorption of nitrogen.

Coverage densities of the ODS chains were determined by CHN analysis. The results of this measurements are presented in Table 1.

RESULTS AND DISCUSSION

As shown by Serpinet /2/, the phase transitions occurring in the chemically-bonded phase may be investigated by means of "inverse" gas chromatography. Such a phase transition is observed as a step on the log V_S = f(1/T) diagrams. The examples of such curves describing the behaviour of C_{18} films bonded to silica gel are given in ref. /1/.

Fig. 1 shows the log V_S vs. 1/T plots. Plot "a" corresponds to the material obtained by bonding ODS to the initial glass, whereas plot "b" presents the same dependence for the sorbent in which ODC was bonded to boron-enriched glass surface. The shape of plot "a" is very similar to those shown by Serpinet:

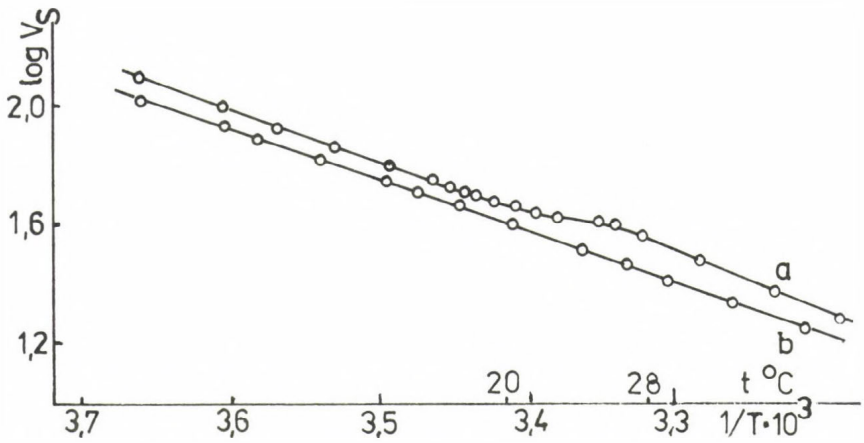

Fig. 1. Log V_S vs. 1/T dependences: a - for glass $0/C_{18}$; b - for glass $5/C_{18}$. Test substance: n-heptane, investigation temperature range: 0 - 90°C

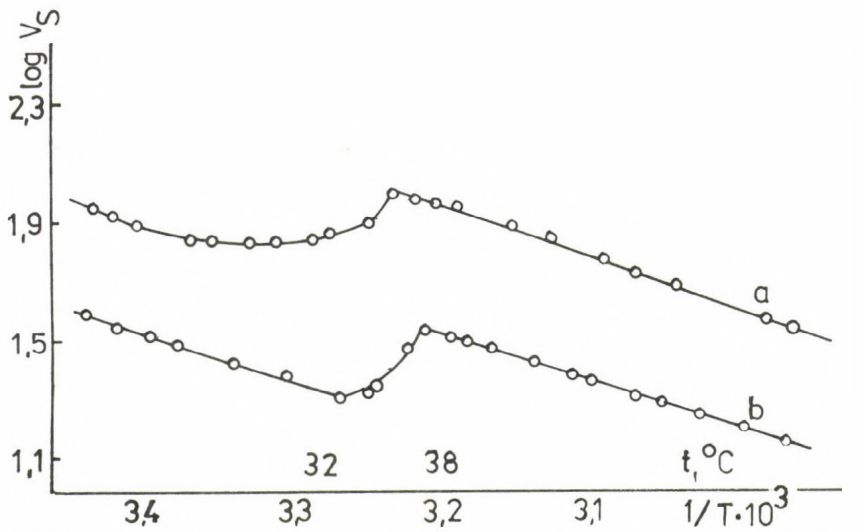

Fig. 2. Log V_S vs. 1/T relationships for: a - glass $5/C_{18}$ covered with 6.2% w/w of n-nonadecane and b - glass $70/C_{18}$ covered with 2% w/w of n-nonadecane

the step in this curve in the 20 - 28°C temperature range testifies the phase transition in the bonded film. It means that the surface properties of the initial porous glass are almost the same as those of silica gel.

The properties of glass surface enriched in boron atoms are different; there are no phase transitions in the film bonded to glass, the surface of which was enriched only for 5 hrs; there is no step in the log V_S = f(1/T) plot, in the investigated temperature range (Fig. 1, curve b). Similarly, no phase transitions are observed for the films bonded to a glass the surface of which was enriched in boron to a higher extent.

A particular property of the alkyl films chemically bonded to the silica gel surface is the possibility to insert the hydrocarbon molecules between the bonded chains. Thus, this mixed film is composed of chemically bonded and physically absorbed aliphatic chains. The mean surface area occupied by one chain is equal to 0.21 nm^2. This is the value characteristic for condensed monolayers of aliphatic ompounds on water surface /10/. In such films phase transitions take place. As a result the film state changes from condensed to expanded state. This behaviour of mixed films is similar to the behaviour of monolayers of amphiphilic compounds on the surface of water or silica gel /10/. Thus, if the gas chromatographic column is filled with a packing composed of silica gel with bonded alkyl film and hydrocarbon deposited on it, two steps may be observed in the log V_S vs. 1/T plots: the first caused by melting of the hydrocarbon and the second resulting from phase transition in the mixed monolayer.

Fig. 2 shows the log V_S vs. 1/T plots for the column packings composed of glass enriched for 5 and 70 hrs, having bonded ODS and covered with 6.2% and 2% w/w of n-nonadecane, respectively. The retention data of n-heptane were investigated in the temperature range of 15 - 90°C.

As shown by Fig. 2 only one step is seen in the presented plots. This step corresponds to the melting of n-nonadecane deposited on the materials studied. It should be stressed that the melting temperature of n-nonadecane (32°C) is shifted to higher values. This phenomenon does not occur in the case of

silica gel with bonded C_{18} film. More details concerning this shift are given in ref. /3/.

The main application of materials with chemically bonded phases is reversed-phase high-performance liquid chromatography. The elution volume in RP HPLC is first of all due to non-specific interactions between the stationary phase and the solute molecules. This interaction may be characterized by the value of the capacity factor, k'. The relationship between $\log(k'/S_{BET})$ vs. n_C, where n_C is the number of carbon atoms in the solute molecules and S_{BET} is the specific surface area of the column packing, is linear for the homologous series of solutes. According to Colin and Guiochon /11/ and our previous investigations /4/ the slope of these plots as well as the values of k'/S_{BET} depend on the density of the coverage. The slope and the k'/S_{BET} values increase with an increase of the C_{18} density.

From the plots presented in Fig. 3 it appears that the most hydrophobic interactions are shown by the material obtained by ODS bonding to the glass surface enriched in boron atoms for 70 hrs. This is confirmed by the data from Table 1. The slope and position of the log (k'/S_{BET}) plot for Lichrosorb Si-100/C_{18} are intermediate between the non-enriched glasses and glasses enriched in boron atoms only for 5 hrs. This is also in agreement with the values of the coverage densities.

Fig. 4 presents the plots of the selectivity coefficient α for the benzene/phenol pair against the percentage of water in the mobile phase. The convexity of such a plot is the measure of homogeneity of the interaction between the solute and the bonded phase. The most convex profile is observed when the retention time is due to the interaction of the solute only with the surface of the bonded phase, or, in the case of mobile phases having a lower polarity, it is due to the solubility of the solute in the mobile phase /12/. Thus, for Lichrosorb Si-100/C_{18} as well as for glass 0 and 5/C_{18} the adsorption phenomena may contribute to the retention mechanism. In the case of glass 70/C_{18} with the most dense C_{18} film the skew of the α vs. $\%H_2O$ plot is the highest. It means that the retention mechanism of the solutes on this material is more homogeneous than on silica gel and glasses 5/C_{18} and 0/C_{18}.

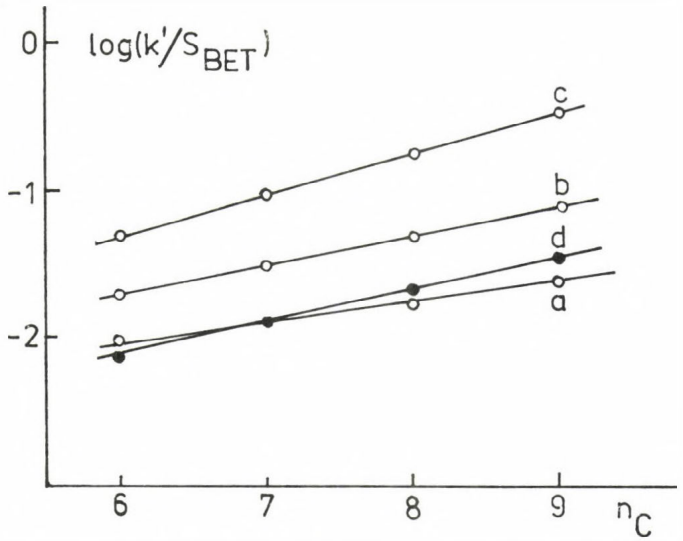

Fig. 3. Log (k'/S_{BET}) vs. the number of carbon atoms n_C for glasses a - $0/C_{18}$, b - $5/C_{18}$, c - $70/C_{18}$ and d - for Lichrosorb Si-$100/C_{18}$. Mobile phase: 60:40 methanol - water

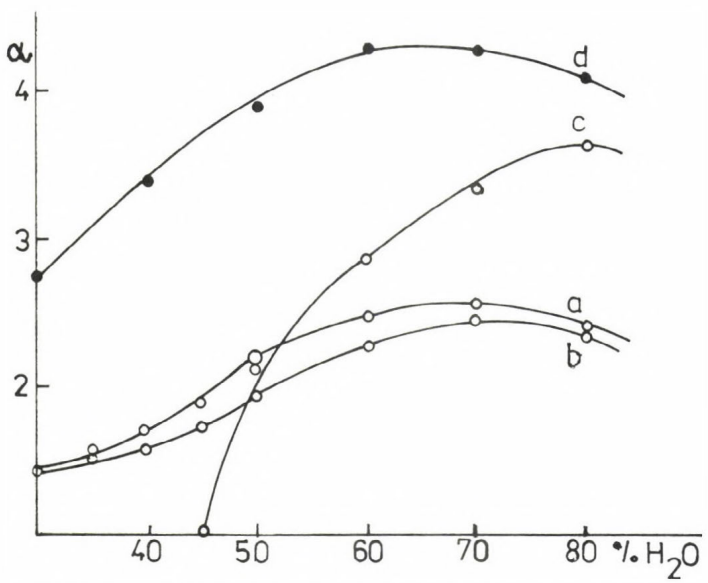

Fig. 4. Selectivity coefficient α of the benzene/phenol pair as a function of the mobile phase composition. "a": glass $0/C_{18}$, "b" glass $5/C_{18}$, "c": glass $70/C_{18}$ and "d": Lichrosorb Si-$100/C_{18}$

Table 2. Comparison of the normalized retention volumes on silica gel and glasses with bonded C_{18} chains

Solute	Normalized retention volume, ml/m^2			
	glass $0/C_{18}$	glass $5/C_{18}$	glass $70/C_{18}$	Lichrosorb Si-100/C_{18}
Benzene	0.017	0.023	0.085	0.011
Toluene	0.019	0.026	0.104	0.015
Ethylbenzene	0.020	0.029	0.140	0.022
Propylbenzene	0.023	0.035	0.206	0.033

In order to compare the HPLC properties of C_{18} films bonded to silica gel and controlled porosity glasses, Table 2 lists the retention volumes related to 1 m^2 of the surface of the material contained in the HPLC columns. Analysing these data it can be seen that the normalized elution volumes are the highest on glass $70/C_{18}$. Because the slope of the log (k'/S_{BET}) vs. n_C plot for this glass is also the highest and the α vs. $\%H_2O$ function is the most convex it is evident that glass $70/C_{18}$ will give the best resolution of the mixtures analysed.

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INFLUENCE OF BORON ENRICHMENT OF CONTROLLED POROUS GLASS SURFACE ON THE ELUTION OF MACROMOLECULES

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INTRODUCTION

Separation in gel permeation chromatography (GPC) is based on the fact that, due to steric exclusion and absence of any energetic interaction, molecules of different size can only penetrate a specific fraction of the total pore volume V_i in the chromatographic column /1/. This leads to the basic eq. (1)

$$V_e = V_o + K_D V_i \qquad (1)$$

where V_e is the elution volume of a given species, K_D is the partition coefficient defining the fraction of the total pore volume V_i which is accessible to any species of a given size, and V_o is the interstitial volume between particles in the column. According to this consideration the separation mechanism of macromolecules is accomplished by the choice of packings and solvents which are suitable for the analysis of polymers of a given size. The proper choice of the column packing is particularly important as the pores dimension of the particles should be adjusted to the magnitudes of the macromolecules analysed /1/.

Among the numerous materials applied as column packings for GPC, controlled porous glasses (CPG) are one of the most interesting /2/. The porous skeleton of CPG consists of SiO_2 (~97%), B_2O_3 (~3%) and Na_2O (~0.5%) /3/. These sorbents are characterized by a high chemical, mechanical and thermal resistance. On

the other hand, almost any porous structure possessing a narrow pore size distribution can be obtained in porous glasses /2/. In addition, these materials can also be considered from the point of view of GPC. This approach results from the presence of electron-accepting boron atoms on the surface /4/. These adsorption centers can cause broadening of the chromatographic peaks and even chemisorption of the molecules analysed /5/. In classical GPC where adsorption effects do not disturb the chromatographic process, the relationship

$$0 < K_D < 1 \qquad (2)$$

obviously holds. If adsorption is observed in the GPC separation, the pure steric exclusion mechanism is disturbed and the partition coefficient frequently exceeds 1 ($K_D > 1$). Such a situation is not convenient for GPC because of the difficulties of chromatography data interpretation. For this reason the chromatographer attempts to work with such a chromatographic system that allows to avoid disadvantageous interactions between the polymer analysed and the sorbent network /6,7,8/.

As it has been confirmed in the literature, the additional thermal treatment of CPG can cause a change of the physicochemical character of its surface /5, 9/. Heating of porous glasses in the temperature range of 670 - 970 K not only leads to a dehydroxylation process of its surface but it also results in the diffusion of the boron atoms remaining in the silica network toward the surface of the glass /10/. As a consequence the adsorption properties of the surface change /5, 9/.

The present paper deals with the influence of the thermal treatment of controlled porous glass on the elution of macromolecules in GPC columns. This problem is examined for a few mobile phases.

EXPERIMENTAL

Materials

The crude material used for the preparation of controlled porous glass was Vycor glass /3, 4/ composed of 7 mol % Na_2O, 23 mol % B_2O_3 and 70 mol % SiO_2 /3, 5/. 10 - 12 μm fraction of this glass was first heated and then leached by 5N H_2SO_4 and 0.5N NaOH according to the procedure described previously /2, 11/. During thermal treatment the Vycor glass separates into two phases. One phase is very rich in silica and insoluble in acids. The second phase, very rich in alkali borate, is soluble in acids and is leached out of the insoluble phase. Leaching in 0.5N NaOH was used in order to remove secondary silica network from the pores of the prepared CPG /2/.

The porous silica lattice of CPG obtained in this procedure still contains residual B_2O_3 and Na_2O. The aim of this paper is to show the influence of the amount of surface boron atoms of CPG applied as column packing upon the elution of macromolecules. For this reason the individual portions of the prepared CPG (hereafter called glass A) were heated at 770 K for 10, 50 and 100 hrs (glass B, C and D respectively). At these conditions the boron atoms remaining in the porous glass network diffuse from the bulk to the surface. The concentration of surface boron atoms is proportional to the time of heating /10/. Such a low heating temperature was used in order to avoid changes in the porosity of materials which underwent different thermal treatments /12/: this is very important from the point of view of GPC.

Measurements

For the chromatographic measurements a Varian Aerograph 4100 liquid chromatograph with a RI detector was employed. This apparatus was additionally equipped with an Orlita AF 10-4 pump and U6K injection system (Waters Assoc.). The flow rate of the mobile phases was monitored to the nearest 0.01 ml. The obtained CPGs were placed in stainless steel columns, 300 mm long x 7.8 mm I.D., using the balanced density slurry techni-

que. Benzene, chloroform and tetrahydrofuran (THF) were employed as the mobile phases. All solvents were carefully dried with molecular sieve 4A and cleaned before use. Ultra-narrow polystyrene standards (PS) (Waters Ass.) were used as the solutes. The interstitial volumes between the particles in the columns were measured by the injection of PS having a molecular weight of $\bar{M}_w$ = 2,610,000. Because the prepared columns might differ in their void volume from one another (not ideal reproducibility in packing - different V_o, and minimal changes in V_i as a result of heating is probable) the partition coefficient and not the elution volumes of the PS were used when investigating the influence of the thermal treatment of CPG on the elution behaviour of the PS. The partition coefficient K_D of a given PS standard was calculated according to the following equation:

$$K_D = \frac{V_e - V_o}{V_t - V_o} \qquad (3)$$

where V_t is the total volume of the mobile phase in the packed column ($V_t = V_o + V_i$). The V_t values were calculated by subtracting the weight of the column filled with dry CPG from the weight of the column filled with CPG and benzene. The drying procedure was carried out at the end of the chromatographic measurements by conditioning the GPC column in a nitrogen stream at 470 K for 3 hrs. In order to avoid concentration effects in the GPC column /13/ the V_e values were calculated by extrapolating the V_e vs. c_i realtionship to $c_i = 0$, where c_i is the concentration of the injected PS solutions.

Pore size distributions of the packings investigated were calculated from mercury porosimetry data. A type 1500 mercury porosimeter (Carlo Erba, Milano, Italy) was applied for this purpose.

RESULTS AND DISCUSSION

Fig. 1 shows the pore size distribution of the prepared sorbents. It appears from Fig. 1 that there are no substantial differences in the mean pore diameter and pore size distribution of the initial glass (A) and the glass heated for the longest

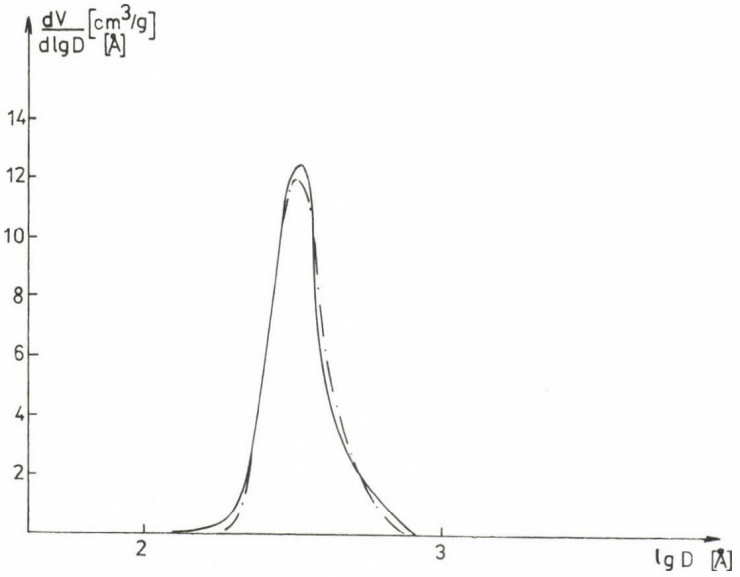

Fig. 1. Pore size distribution functions for glass A and glass D

time (D). CPGs are more thermally resistant in relation to silica gel. The 773 K temperature does not change the porosity of the glass obtained, similarly as in the case of the original Vycor glass 7930 /12/.

Table 1 lists the elution volumes (V_e) and partition coefficients (K_D) of polystyrene standards on the columns filled with prepared glasses. Table 1 also gives the total volumes V_t of these columns. Comparing the data presented in Table 1 it is evident that the behaviour of the PS standards is different on the individual glasses and depends on the type of mobile phase. This behaviour is better observed in Figs 2, 3 and 4, where

Table 1. Elution volumes and partition coefficients of PS on columns packed with glasses A, B, C and D

$\bar{M}_w$ of PS	Glass A		Glass B		Glass C		Glass D	
	V_e(ml)	K_D	V_e(ml)	K_D	V_e(ml)	K_D	V_e(ml)	K_d
a Mobile phase: chloroform								
2 610 000	6.00	0.00	5.95	0.00	6.10	0.00	6.09	0.00
670 000	6.00	0.00	5.94	0.00	6.10	0.00	6.09	0.00
390 000	6.33	0.07	6.15	0.04	6.25	0.03	6.19	0.02
200 000	7.63	0.33	7.35	0.28	7.37	0.26	7.35	0.25
111 000	9.24	0.65	8.81	0.57	8.80	0.55	8.77	0.53
33 000	10.54	0.91	10.21	0.85	10.12	0.82	10.18	0.81
20 800	11.02	1.00	10.56	0.92	10.41	0.88	10.53	0.88
8 000	11.41	1.08	10.91	0.99	10.76	0.95	10.84	0.94
3 600	11.57	1.11	11.01	1.01	10.85	0.97	10.94	0.96
2 100	11.61	1.12	11.06	1.02	10.95	0.99	10.99	0.97
styrene	11.78	1.15	11.11	1.03	11.00	1.00	11.09	0.99
b Mobile phase: benzene								
2 610 000	6.10	0.00	6.00	0.00	6.08	0.00	6.05	0.00
670 000	6.10	0.00	6.20	0.04	6.23	0.03	6.05	0.00
390 000	6.44	0.07	6.55	0.11	6.62	0.11	6.36	0.06
200 000	7.77	0.34	7.88	0.38	8.00	0.39	7.73	0.33
111 000	9.49	0.69	9.62	0.73	9.72	0.74	9.51	0.68
33 000	10.76	0.95	10.96	1.00	11.10	1.02	10.89	0.95
20 800	11.30	1.06	11.46	1.10	11.54	1.11	11.39	1.05
8 000	11.55	1.11	11.70	1.15	11.84	1.17	11.80	1.13
3 600	11.75	1.15	11.90	1.19	11.98	1.20	11.95	1.16
2 100	11.84	1.17	12.05	1.22	12.18	1.24	12.10	1.19
styrene	12.09	1.12	12.40	1.29	12.53	1.31	12.41	1.25
c Mobile phase: tetrahydrofuran								
2 610 000	6.03	0.00	6.05	0.00	6.04	0.00	6.00	0.00
670 000	6.03	0.00	6.15	0.02	6.04	0.00	6.00	0.00
390 000	6.38	0.07	6.44	0.08	6.29	0.05	6.26	0.05
200 000	7.67	0.33	7.77	0.35	7.63	0.32	7.54	0.30
111 000	9.37	0.67	9.44	0.69	9.26	0.65	9.29	0.64
33 000	10.71	0.94	10.76	0.96	10.60	0.92	10.63	0.90
20 800	11.11	1.02	11.16	1.04	10.95	0.99	11.04	0.98
8 000	11.51	1.10	11.55	1.12	11.35	1.07	11.45	1.06
3 600	11.66	1.13	11.70	1.15	11.50	1.10	11.55	1.08
2 100	11.71	1.14	11.75	1.16	11.55	1.11	11.55	1.08
styrene	11.86	1.17	11.94	1.20	11.64	1.13	11.65	1.10
	V_t = 11.01		V_t = 10.96		V_t = 11.00		V_t = 11.14	

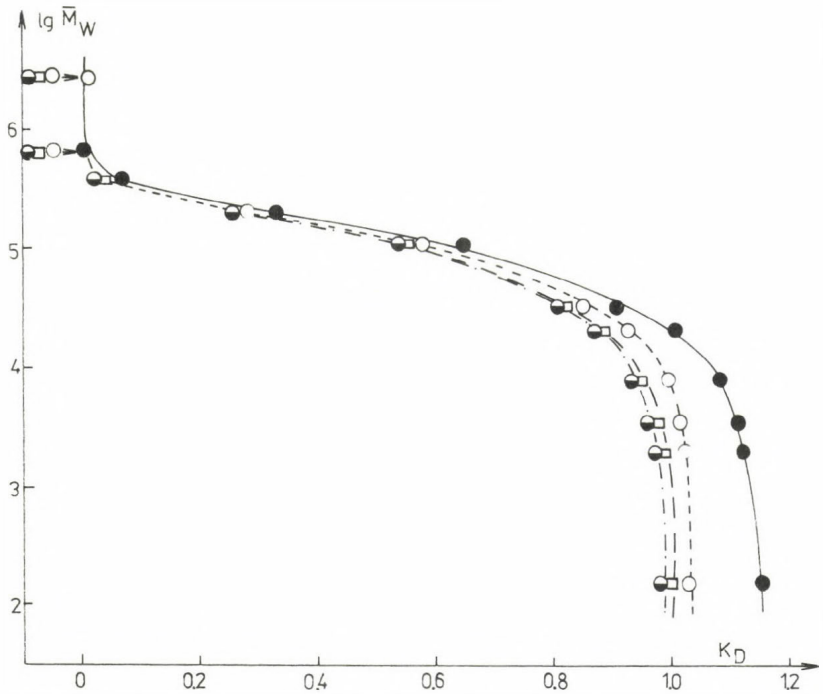

Fig. 2. Partition coefficients of ultra narrow PS standards having different molecular weights ($\bar{M}_W$) on columns filled with: glass A (——●——); glass B (---o---); glass C (– –□– –); glass D (–·◒·–). Mobile phase: chloroform

the molecular weight is plotted against the partition coefficient dependences obtained on the different glasses.
Both the data presented in Table 1a and the plots shown in Fig. 2, indicate a gradual decrease of the partition coefficients of the individual PS standards with the increase of the time of the thermal treatment of CPG, when using $CHCl_3$ as the mobile phase. When benzene is employed as the mobile phase (Table 1b, Fig. 3), an increase is seen in the lg $\bar{M}_W = f(K_D)$ function from glass A to glass C, and then a drop for the column packed with glass D, thermally treated for a very long time. The K_D values of the given PS on the column with glass D are almost the same as on the column with glass A. In the case of THF the shape of lg $\bar{M}_W$ vs. K_D relationships is slightly different. Table 1c and

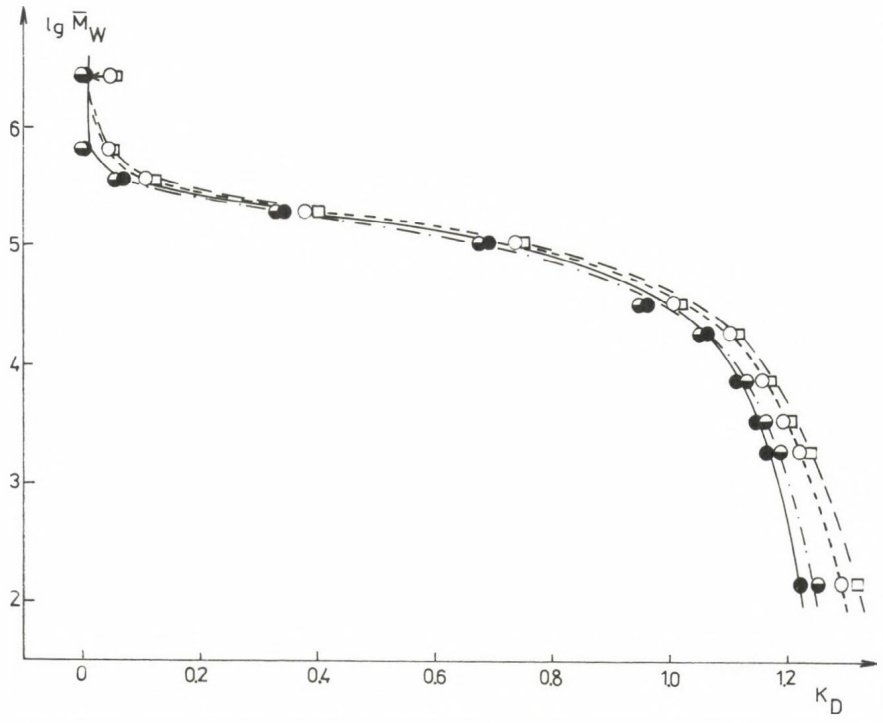

Fig. 3. Partition coefficients of ultra narrow PS standards having different molecular weights (M_w) on columns filled with: glass A (——●——); glass B (---o---); glass C (– –□– –); glass D (–·–●–·–). Mobile phase: benzene

Fig. 4 show an increase in K_D from glass A to B and a decrease in K_D for other materials. Studying the influence of the type of mobile phase on the same column, the highest elution volumes can be seen in benzene and the lowest in chloroform. The same dependence was previously observed /14/.

As mentioned before, the thermal treatment of CPGs causes an enrichment of their surface in boron atoms. The presence of electron-accepting boron atoms on the CPG surface can both diminish or increase the adsorption properties of the surface with respect to the analysed molecules, depending on the system: mobile phase/compound. The adsorption properties are reduced when the mobile phase molecules form a compact and dense film

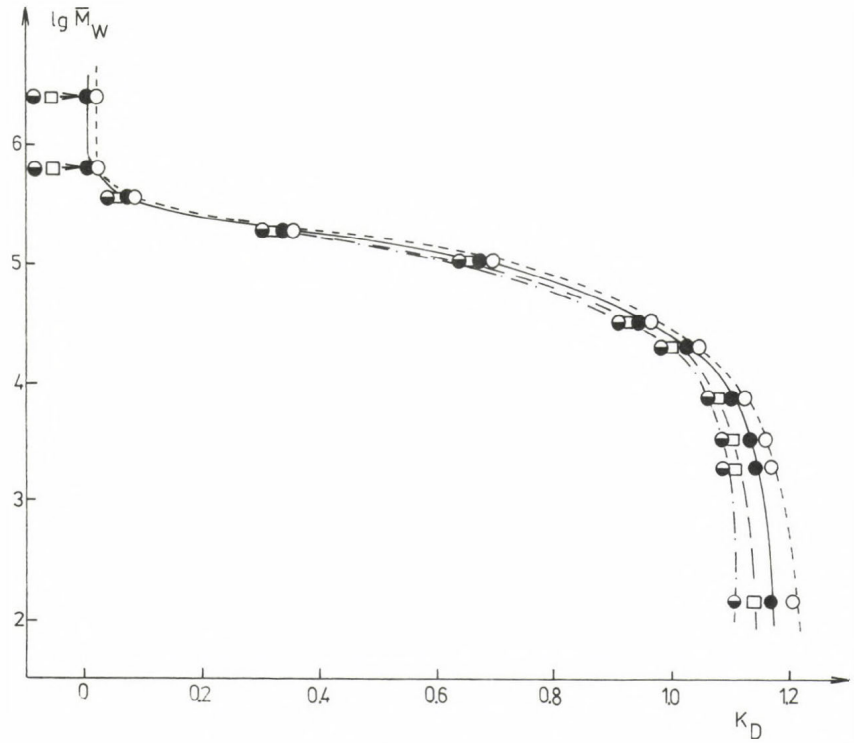

Fig. 4. Partition coefficients of ultra-narrow PS standards having different molecular weights ($\bar{M}_W$) on columns filled with: glass A (——●——); glass B (---o---); glass C (– –□– –); glass D (–·–●–·–). Mobile phase: THF

on the surface of the sorbent, decreasing the interaction between the adsorptive centers of the surface and the molecules analysed (e.g. molecules of the mobile phase possessing electron-donor atoms and electron-acceptor boron atoms from the surface). Such is probably the situation in the case of chloroform. It is known from previous data /5/ that the thermal treatment of CPG leads to a gradual increase of $CHCl_3$ adsorption. In consequence the increase of surface boron atom concentration causes the formation of a more and more compact film screening the interaction between PS and boron-enriched CPG surface. As a result the partition coefficient of the macromolecules decreases and,

in the case of glass C and D, reaches the value of unity; the separation mechanism is purely steric (Fig. 2, Table 1a).

A reverse situation is true for benzene. As it was previously reported /5/ heated CPG shows lower adsorption properties at short heating times for benzene molecules than the initial glass. A very long thermal treatment increases the adsorption properties of CPG to values close to those characteristic for the initial glass. Taking into account this consideration and examining the plots in Fig. 3 as well as the values in Table 1b, it can be seen that the surface benzene film does not screen the PS/surface interaction as strongly as in the case of chloroform. In benzene used as the mobile phase the interactions between the PS shells and the boron-enriched surface initially increase from glass A to C, and then they fall from glass C to D and are similar to those obtained for glass A. The presented data agree with the adsorption isotherms for benzene /5/.

The situation is different for THF used as the mobile phase (Fig. 4, Table 1c). At the beginning of the thermal treatment the K_D values of PS will first slightly increase and then decrease. Similarly to chloroform, THF contains electron-donor atoms, and strongly interacts with the boron-enriched CPG surface /5/. Chloroform has three electron-rich chlorine atoms, whereas THF has one ether type oxygen atom. It is probable that chloroform interacts with boron-enriched CPG via its three chlorine atoms and take up a tripod-like configuration. Considering such a model, one chloroform molecule will block a larger surface area than one THF molecule.

CONCLUSIONS

Concluding, the enrichment of glass surface in boron atoms decreases the elution volume of polystyrene standards if a mobile phase possessing electron-donor properties is used. The data presented illustrate that, besides the silanization process /6/ and the addition of substances to the mobile phase which block the adsorptive centers on the CPG surface /8/, the enrichment of CPG surface in boron atoms seems to be a con-

venient and useful method to decrease the interaction between the macromolecules and the surface.

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APPLICATION OF AIRCOMP 16 PERSONAL COMPUTER TO THE QUALITY CONTROL OF HPLC COLUMNS

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SUMMARY

The AIRCOMP 16, the smallest home computer built in Hungary (Personal G.T. Budaörs, Hungary) has been investigated for the quality control of LC packing materials and packed columns. The 16k free memory area is enough to solve several calculating problems (e.g., measurement of pore size distribution, theoretical plate height - linear velocity relationship.)

INTRODUCTION

HPLC packing materials and packed columns are often characterized by the porosity and efficiency of the column. In the practice this means that a large amount of measured data must be worked up. It is mathematically simple, but to achieve good, statistically correct results computer methods are preferred to characterize the chromatographic columns.

DESCRIPTION OF METHODS

The heart of the AIRCOMP computer is a Z 80A microprocessor. The free area is of 16115 bytes. Accordingly nearly full of the memory area can be used as RAM BASIC area.

For porosity measurements we use the size exclusion method elaborated by Halász and Martin /1/. The specific pore volume

($V_{p,sp}$) is calculated from the retention volume difference between a samll molecule (V_{max}; benzene, ϕ = 0.8 nm) and a very large molecule excluded from all pores (V_{min}; polystyrene, $M_w = 3.7 \cdot 10^6$, ϕ = 453 nm):

$$V_{p,sp} = \frac{V_{max} - V_{min}}{m}$$

(m: weight of the packing material in the column)

To evaluate pore size distribution and average pore diameter ($\bar{\phi}$) elution volumes for a series of narrow dispersion molecular weight polystyrenes, having a molecular diameter between 0.8 and 453 nm, are determined.

For the determination of the specific surface area (S) we use Kelvin's formula /2/:

$$S = \frac{4 \cdot V_{p,sp}}{\bar{\phi}}$$

Interstitial porosity (ε_o), pore porosity (ε_p), and total porosity (ε_T) are expressed by the following relationships:

$$\varepsilon_o = \frac{V_{min}}{V_c} \; ; \quad \varepsilon_p = \frac{V_{max} - V_{min}}{V_c} \; ; \quad \varepsilon_T = \frac{V_{max}}{V_c}$$

(V_c is the volume of the empty column.)

We inject benzene not only to determine the dead volume of the column, but also as the standard for controlling the flow uniformity, by all injections.

The computing scheme is given in Fig. 1. In step 3 all retention data entered in step 2 and retentions (k') of various polystyrenes, relative retention of polystyrenes to benzene, and the percentage pore volume ($V_{\phi=0.8}$ = 100%; $V_{\phi=453}$ = 0%) are visualized in a table. Typing errors can be corrected.

In step 4 a so-called "Gaussian field" is plotted. If pore distribution is of a Gaussian type the relative retention vs.

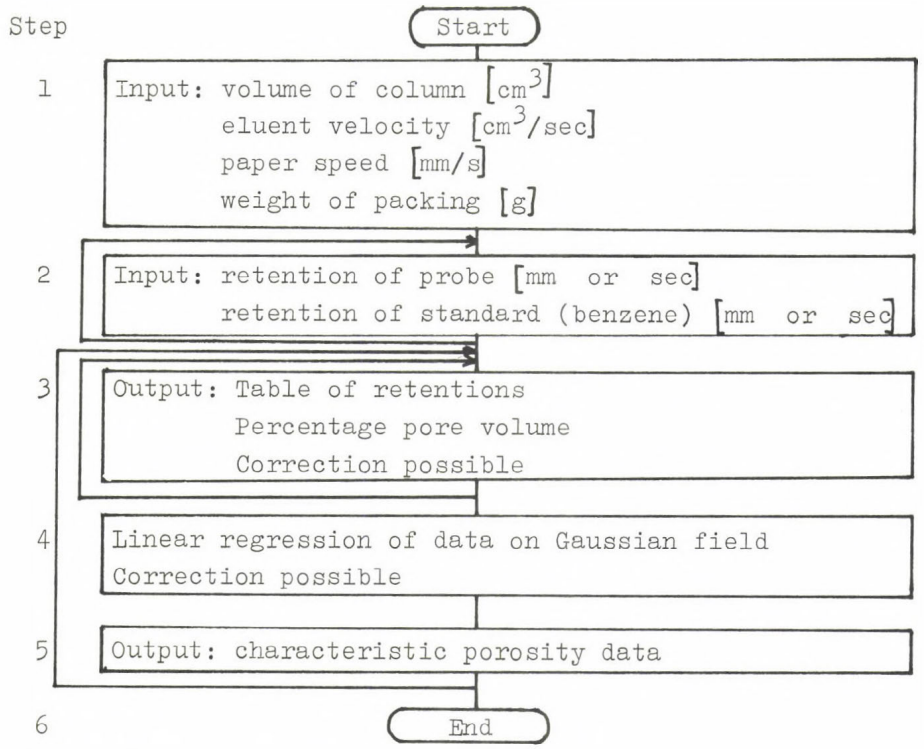

Fig. 1. Simplified Flow Chart for porosity measurement

molecular diameter plot should be a straight line. (This line can be calculated by linear regression.) The diameter corresponding to 50% pore volume is the average pore diameter ($\bar{\phi}$). As the pore size distribution is only nearly Gaussian type, the points may deviate from the straight line. Correction is possible; in a second run this data can be left out. Results are: $\bar{\phi}$ [nm]; $V_{p,sp}$[cm^3/g]; S [m^2/g]; ε_o; ε_p; ε_T and their standard deviations.

A second program calculates the terms A, B and C of the van Deemter equation /3/ and plots the theoretical plate height (H) versus the linear velocity (Fig. 2). The required input data are:

- number of H-u curves: G, max. 3. (To characterize an LC column determination of three H-u curves by different k' are usually enough.);

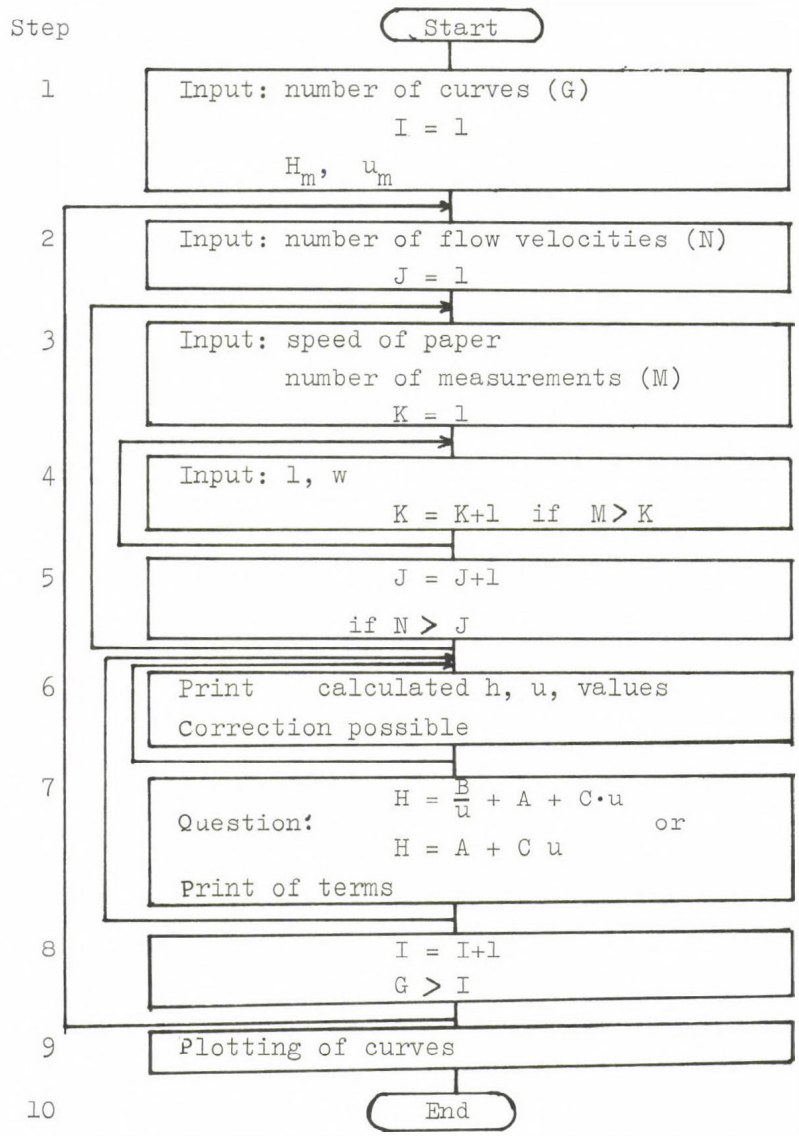

Fig. 2. Simplified Flow Chart for H-u relationship measurement

- H_m and u_m (These are the maximum H and u values on the coordinates by plotting the curves.);
- number of linear flow velocities (N, max. 14);
- number of measurements by one flow velocity (M);
- speed of paper (mm/s);
- retention (, mm), because the speed of registration paper is given;
- bandwidth of peak (w, mm) measured at the base line, or at half peak height.

Explanation to step 7: we measure the H-u relationship often only at relatively high linear velocities, so we cannot determine the B term. In this case we use a simple linear regression, a so called "short form" of the van Deemter equation.

Explanation to step 9: all three curves can be plotted in one coordinate system for better visualization.

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DRUGS, METABOLITES, BIOLOGICALLY ACTIVE COMPOUNDS, ENDOGENOUS SUBSTANCES

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USE OF HPLC IN CHARACTERIZING THE EFFECTS OF AF64A, A POTENTIAL CHOLINERGIC NEUROTOXIN

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INTRODUCTION

In the past few years, high-performance liquid chromatography with electrochemical detection (HPLC-ED) has become a powerful and valuable tool in neurochemistry. One of the most widely used applications of HPLC-ED has been for the measurement of the biogenic amines noradrenaline (NA), dopamine (DA), serotonin (5HT) and their metabolites. Recently, the specificity of this method has been further improved by the introduction of electrochemical cells with dual working electrodes that allow simultaneous analysis of the eluent at different potentials, thus making it possible to screen out interfering substances that may have similar chromatographic, but different electrochemical, properties /1/. One application of this is the redox mode with coulometric electrodes; electroactive compounds are reduced at the first electrode and their hydrolysis products are oxidized at the second /1/. This has the advantage of limiting the number of compounds that will be detected in a biological sample since only those compounds which are able to react reversibly will produce a signal. Another recent development in HPLC-ED has been a method for assay of acetylcholine (ACh) using a post-column conversion of ACh and choline to betaine and H_2O_2, which is detected with a platinum electrode /2/. In the present study we have applied both of these recent developments to a neurochemical problem: the evaluation of a possible animal model for Alzheimer's disease.

Alzheimer's disease is characterized by a major degeneration of the cholinergic pathway from the nucleus basalis of Meynert in the basal forebrain to the cerebral cortex /3/. Loss of this pathway results in a severe , progressive loss in memory and cognitive function. A number of therapies have been tried in an attempt to improve cholinergic function and alleviate the symptoms of Alzheimer's disease /3/. These therapies have provided few consistent beneficial effects. Thus it seems imperative that the actions of possible therapeutic agents be tested in animal models where cholinergic function in the central nervous system is severely impaired.

Unfortunately, there has been no good animal model for Alzheimer's disease or for any condition in which the cholinergic system is specifically impaired. However, a neurotoxin which is selective for cholinergic nerves could be used to provide such an animal model. Recently, ethylcholine mustard arizidinium ion (AF64A) has been shown to cause a reduction in markers for central cholinergic nerve terminals /4/. Intracerebroventricular (i.c.v.) administration of AF64A in the mouse produced dramatic reductions in cholinergic indices such as ACh levels, high affinity choline uptake (HAChT) and choline acetyltransferase (ChAT) activity /4, 5/. Similar effects were seen when AF64A was injected directly into the hippocampus or striatum of the rat /6, 7/. These studies indicate that AF64A reduces cholinergic activity in the central nervous system through death of, or damage to, nerve terminals.

It is important to determine whether AF64A causes an effect specific only to cholinergic nerves, like that of 6-hydroxy-dopamine for catecholamine-containing neurons. This was investigated in the studies where AF64A was injected into the hippocampus or striatum; markers for noradrenergic, serotonergic, dopaminergic and GABAergic neurons were not affected by AF64A /6, 7/. However, in other studies in which AF64A was injected into the substantia innominata /8/ or substantia nigra /9/, nonspecific effects were described. It is likely that the selectivity of AF64A depends upon the dose and the route of administration.

Therefore, one of the aims of the present study was to verify the specificity of the effect of AF64A injected i.c.v. in the rat. HPLC-ED in the redox mode was used to measure NA, DA, and 5HT levels in various brain regions after treatment with AF64A. We found that injection of 10 nmol AF64A into one lateral ventricle caused reversible changes in brain amine levels, suggesting that when administered in such a way as to reach a high local concentration, AF64A can exert nonspecific toxic effects.

Another aim of this study was to assess the effect of AF64A on the functional state of central cholinergic neurons, by measuring the release of endogenous ACh from three different brain regions and at three different times after AF64A administration. We found that ACh release from the hippocampus was markedly reduced 2, 4, and 7 days after treatment with 10 nmol AF64A, but ACh release from striatum or frontal cortex was not affected. Bioassay on the guinea-pig ileum /10/ was used to measure release because of the high sensitivity of this method compared to others used for measuring ACh /11/. This sensitivity is necessary to detect the small amounts of ACh released, particularly under resting conditions. The disadvantage of bioassay is that it lacks specificity. Therefore, we characterized the spasmogenic substance released from rat hippocampus by HPLC separation and comparison of the elution pattern of released material with that authentic ACh standards, as described by Vizi et al. /12/. We found that the spasmogenic substance eluted in exactly the same pattern as did the ACh standard, no other peaks were seen, and the recovery of spasmogenic activity in standards and in samples was the same, indicating that all of the material released from hippocampus and measured on the guinea pig ileum could be accounted for as ACh.

METHODS

Preparation and Administration of AF64A

AF64A was prepared by basic hydrolysis of 1 mM acetylethylcholine mustard and subsequent arizidinium ion formation as

described by Fischer et al. /5/. The pH was adjusted to 11.3-11.7 with 10 N NaOH and the solution was stirred at room temperature for 30 min, then the pH was reduced to 7.4 with concentrated HCl. AF64A solutions were prepared immediately before administration and NaCl was added to a final concentration of 0.9%. Male Wistar rats (140-150 g) were anaesthetized with pentobarbital (40 mg/kg i.p.) and placed into a small animal stereotaxic frame. A 30 gauge cannula was inserted through the skull at coordinates A-1.5 mm, L+1.5 mm, V-4.0 mm. Saline or AF64A (10 nmol) was injected in a volume of 10 µl over a 3 min period. Animals were housed 4 per cage on a 12-hour light/dark cycle with free access to food and water and were killed by decapitation or by head-focussed microwave irradiation (5 kW, 1.8 sec, Gerling-Moore microwave generator) 2, 4, or 7 days after treatment.

Measurement of ACh Release

Endogenous ACh release was determined as described by Vizi et al. /13/. The pair of hippocampi, striata or frontal cortices from each rat were dissected and placed into 5 ml double walled organ baths thermostated at 37^{o}, continuously oxygenated with 95% O_2 : 5% CO_2, and containing 2 ml of Krebs solution of the following composition (mM): NaCl 113, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 11.5, and eserine sulfate 0.0062. After 1 hour pre-incubation, the Krebs solution was changed at 10 min intervals; in the 3rd and 4th 10 min periods, Krebs solution containing $2x10^{-5}$M ouabain was added to stimulate ACh release /14/. At the end of each 10 min period, the bath solution was collected and assayed for ACh by bioassay on the guinea-pig ileum as described by Paton and Vizi /10/.

Identification of ACh by HPLC

Hippocampi from 4 rats were pooled in a 5 ml organ bath and incubated in 2 ml Krebs solution as described above. The bath contents from the two 10 min stimulation periods in the presence of ouabain were collected, pooled and lyophilized. The

samples were re-dissolved in 100 µl of 0.01 Na acetate/citric acid, pH 4, and 20 µl was injected into an HPLC consisting of a Milton Roy pump, Rheodyne 7125 injector with a 20 µl loop, and a 10 cm Hamilton PRP-10 column (Biotronik Wissenschaftliche Gerate GmbH, Frankfurt am Main, West Germany and Kontron Analytic, Vienna, Austria). Mobile phase consisted of 0.025 M Tris-maleate adjusted to pH 7 with NaOH, containing 1 mM tetraethylammonium iodide. Flow rate was 0.75 ml/min. Fifteen 1 min fractions were collected and assayed for ACh on the guinea-pig ileum. The elution of ACh in hippocampal incubation fluid was compared with that of ACh standards. In addition, the standards and samples were diluted 20 : 750 and assayed on the guinea-pig ileum. Recovery was estimated by comparing the spasmogenic activity eluted from the column with that in the diluted standards and samples.

Determination of amine contents by HPLC-ED

Striata, frontal cortices and hippocampi were dissected from rats which had been killed with head-focussed microwave irradiation, weighed, and homogenized by sonication in 100 µl of 0.2 N perchloric acid with 0.11 M ascorbic acid, then centrifuged at 10 000 g for 20 min. A 20 µl aliquot of the clear supernatant was injected into the HPLC. The HPLC system consisted of a Biotronik BT 3020 high pressure pump with pulse dampener and Rheodyne 7125 injector with 20 µl loop, a 15 cm Nucleosil C_{18} reversed-phase column with 5 µm particles (Bischoff Analysentechnik) and an ESA (Environmental Sciences Associates Inc., Bedford, MA, USA) electrochemical detector with an ESA Model 5020 guard cell at a potential of +0.6 V and a Model 5010 dual electrode analytical cell with porous graphite working electrodes at potentials of -0.5 V and +0.3 V, respectively, versus palladium reference electrodes. Signals from the electrochemical detector were monitored on a two-channel chart recorder. Mobile phase consisted of 0.1 M sodium acetate/citric acid, pH 4, containing 0.4 mM sodium octyl sulfate. The mobile phase was filtered twice through 0.45 µm Millipore filters then degassed in an ultrasonic generator.

For determination of NA, 2% MeOH was added to the mobile phase, and for estimation of DA and 5HT, 12% MeOH was used. Standards were diluted in 0.2 M perchloric acid with 0.11 mM ascorbic acid from stock solution stored frozen in 1 N HCl. The detector response was linear in the range of 1.5-200 pmol NA, DA, or 5HT/20 µl. Retention times were: NA 7.5 min, DA 5.8 min 5HT 15 min. The amine contents of the samples were quantitated by comparing the peak heights with standard curves and were expressed per gram of tissue.

RESULTS

Identification of ACh by HPLC

Figure 1 shows the elution profiles of an ACh standard and of a sample of hippocampal perfusate. Spasmogenic activity was seen in fractions 3, 4, and 5. No detectable activity was found in any of the other 12 fractions. Recovery of the ACh standard was 81% and that of the hippocampal effluent was 77%. This result confirms the assumption that the spasmogenic activity measured by bioassay in hippocampal perfusates was ACh and that there were no interfering substances.

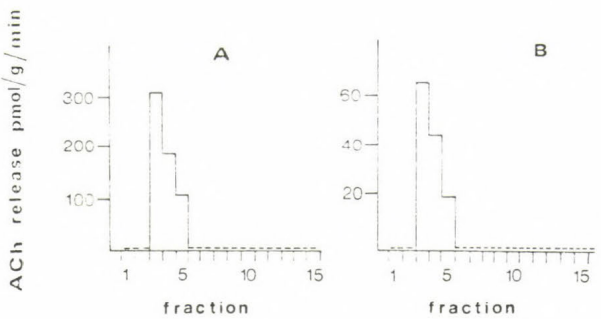

Fig. 1. Identification of ACh by HPLC.
20 µl of ACh standard or hippocampal perfusate was injected into the HPLC and the ACh content of 1 min fractions was determined by bioassay. HPLC conditions as described in Methods.
A: ACh standard, B: Hippocampal incubation fluid

Effect of AF64A on ACh release

As shown in Figure 2, unilateral injection of 10 nmol AF64A decreased the ouabain stimulated release of ACh from hippocampus 2, 4, and 7 days after treatment. The effect of AF64A was most pronounced on the fourth day. Only the release in response to stimulation was decreased; the resting release of ACh was not affected significantly.

The action of AF64A on ACh release was limited to the hippocampus. Ouabain stimulated ACh release from the striatum or frontal cortex was not changed 4 days after AF64A treatment (Table 1).

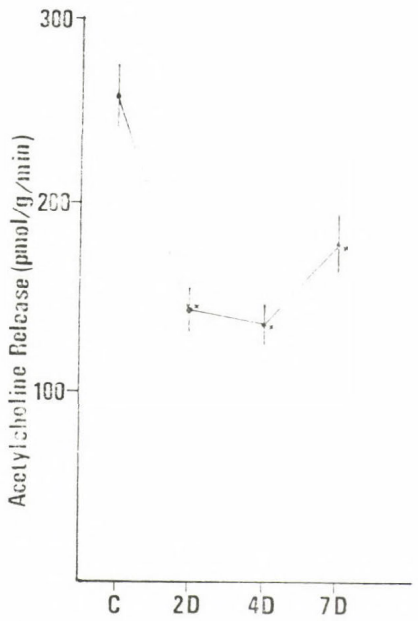

Fig. 2. Effect of 10 nmol AF64A on ACh release from hippocampus. C: control. 2D, 4D, 7D: days after treatment with AF64A. ACh release was measured by bioassay from hippocampi from control and AF64A treated rats. Values shown are the ACh content in the incubation fluid during the second 10 min stimulation period with ouabain, 2×10^{-5}M. *$p<0.05$, Student's two tailed t test. Number of animals 4.

Table 1. Effect of 10 nmol AF64A on ACh Release from Striatum and Frontal Cortex

	ACh Release (pmol/g/min)
Striatum	
Saline	1580.82±110.53
AF64A	1477.56±198.25
Frontal Cortex	
Saline	199.25±17.15
AF64A	229.53±23.77

ACh release was measured by bioassay from striata or frontal cortices of rats which received i.c.v. injections of 10 µl saline or 10 nmol AF64A four days prior to experiments. n=4 in all groups. Values shown are ACh content in the incubation fluid during the second 10 min stimulation period with ouabain, $2x10^{-5}$M.

Effect of AF64A on Amine Contents

The amine contents measured in striata, hippocampi and frontal cortices from control and AF64A treated rats are shown in Figure 3. There was no significant difference in the amine contents measured in the injected and non-injected sides (data not shown). Nonspecific effects of AF64A were seen in all brain regions, particularly in the hippocampus where NA, DA and 5HT were all reduced significantly 4 days after treatment. This effect was reversible; the amine contents had recovered and were not significantly different from control on the seventh day. AF64A affected serotonergic systems more than it did the others. 5HT levels were reduced in all three brain regions 4 days after AF64A administration. Again, the effect was reversible and the 5HT levels recovered by 7 days. In the frontal cortex, there was a significant increase in DA levels 7 days after treatment.

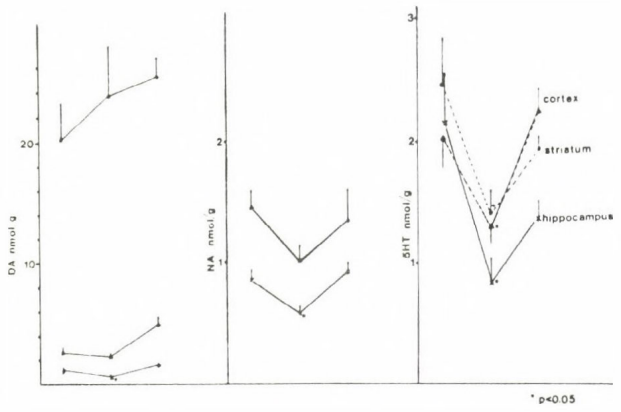

Fig. 3. Effect of 10 nmol AF64A on amine content.
C: control. 4D, 7D: 4 and 7 days after AF64A treatment. Amine contents were determined by HPLC-ED as described in Methods in striata, hippocampi and frontal cortices dissected from rats which had received i.c.v. injections of saline or 10 nmol AF64A. Tissues were pooled from injected and non-injected sides. n=4 in each group.
*$p < 0.05$, Student's two tailed t test.

DISCUSSION

The results presented in this study indicate that (a) the release of endogenous ACh from brain tissue can be measured by bioassay and characterized by HPLC separation. (b) The ouabain-stimulated ACh release so determined is markedly reduced in hippocampus after i.c.v. administration of AF64A, a cholinergic neurotoxin, indicating that this compound produces sufficient disruption of ACh-containing nerve terminals to affect function as represented by release. (c) The effects of AF64A were most pronounced in hippocampus; reductions in striatal or cortical ACh release were not observed. (d) Amine contents were reduced on the fourth day after treatment, a time when the effects on ACh release were maximal.

This change in amine contents probably did not result from destruction of amine-containing nerve terminals, because there was a recovery of amine levels by the seventh day after treatment.

An important aspect of this study was the identification of the substance released from brain tissue as ACh. For this purpose, the chromatographic conditions which have been described for ACh were used /2, 12, 15, 16/. With the Hamilton PRP-10 column, ACh was eluted in the 3rd, 4th, and 5th fractions. No other peaks of spasmogenic activity were seen in either standards or samples, and the elution pattern and recovery of samples was identical with that of standards. Were there any interfering compounds, this would not be the case. As additional identification, the experiment was repeated with a Nucleosil C_{18} column and mobile phase consisting of 0.1 M Na acetate/citric acid, pH 4 and 1 mM tetraethylammonium. Again, the elution and recovery of standards and samples were the same. In another study, the substance released from striatum by ouabain was identified as ACh by HPLC and bioassay /12/. Furthermore, the spasmogenic activity was abolished by atropine pretreatment of the guinea-pig ileum (data not shown). Thus, three checks (atropine sensitivity, elution pattern, and recovery) have served to identify the compound measured by bioassay in these experiments as ACh. The use of HPLC lends a new measure of specificity to the bioassay technique.

Several previous studies have shown that administration of AF64A, either i.c.v. in mouse or directly into tissues in rat, results in a loss of cholinergic markers such as ACh content, HAChT and ChAT activity /4-7/. In the present study the ouabain-stimulated ACh release was measured as an index of the functional state of cholinergic neurons. Administration of AF64A resulted in a marked decrease in the stimulation induced release of ACh from hippocampal tissue within two days of injection (Figure 2), an effect which was most pronounced 4 days after treatment but was still significant on the seventh day. This effect was seen only in the hippocampus and not in striatum or frontal cortex. Other studies in which AF64A was given i.c.v. to mice or rats have shown that other markers, such as ACh levels, are decreased earlier and to a greater extent in hippocampus that in striatum or cortex /5, 17/. At present it is not known why the cells of the hippocampus are more susceptible to the effects of AF64A. Indeed, in vitro experiments /17/

demonstrate that one of the acute effects of AF64A, an increase in ACh release, is seen only in the hippocampus and not in striatum or cerebral cortex. Another possibility, of course, is that AF64A diffuses more rapidly from the lateral ventricle to the hippocampus than to the other areas tested; the answer to this question awaits the development of radiolabelled AF64A.

However, even the non-specific effects of AF64A were more pronounced in the hippocampus: NA, DA and 5HT levels were all reduced 4 days after treatment. Serotonin contents in the striatum and frontal cortex were also decreased on the fourth day after injection of AF64A. It is not likely, though, that AF64A destroyed these neurons, for the amine levels had recovered and were not significantly different from control by the seventh day following administration of AF64A. It seems unlikely that changes in amine levels resulting from cell damage would be so rapidly reversed. Even within two days after treatment, AF64A had caused sufficient disruption of hippocampal cholinergic neurons that the release of transmitter was reduced. This hypoactivity of the cholinergic system could possibly lead to changes in the interactions between aminergic and cholinergic neurons. This might be reflected in changes of the levels of amines until the two systems had readjusted. Perhaps a study of the synthesis rate of the amines following AF64A might yield valuable information on the interaction between aminergic and cholinergic systems.

A destructive effect of AF64A on aminergic neurons also seems surprising in light of studies showing that direct injection of AF64A into the hippocampus or striatum did not affect noradrenergic, dopaminergic, serotonergic, or GABAergic markers /6, 7/. The dose and route of administration, however, are certainly important. In the studies mentioned above, 2 nmol AF64A was injected into the hippocampus and 8 nmol into the striatum, whereas in this study 10 nmol was injected into the lateral ventricle, which may have resulted in a high local concentration of AF64A in the hippocampus. In fact, studies currently in progress /17/ indicate thatnon-specific effects can be avoided if 5 nmol AF64A is injected bilaterally. There have been two other reports of non-specific effects of AF64A, fol-

lowing injection into cell body areas of the substantia nigra /9/ or the substantia innominata /8/. This was unexpected, since as the action of this compound almost certainly involves interaction with the high affinity choline uptake system /18/, it should act preferentially at cholinergic nerve terminals and not at cell bodies. However, it appears that, at a high enough concentration, AF64A is able to damage non-cholinergic cells, possibly through alkylation of membrane components. It is therefore advisable to exercise caution and to determine the specificity of any new dose or method of administration of AF64A.

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RAPID AND RELIABLE HPLC METHOD FOR ROUTINE MONITORING OF GLIBENCLAMIDE CONCENTRATION IN SERUM

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SUMMARY

A sensitive high-performance liquid chromatographic method on a reversed phase column has been developed with UV-detection for glibenclamide from serum, which is proposed for the routine monitoring of the drug.

The method involves salt precipitation of serum proteins followed by ethyl acetate extraction. The detection limit is 10-12 ng from 1 ml serum extracted. The recovery is 70-75%.

The method was applied to single-dose studies of two different preparations of glibenclamide. The results have important implications in the determinations of bioavailability and pharmacokinetics of the drugs.

INTRODUCTION

Sulfonylurea drugs are widely used in the treatment of diabetes mellitus of the maturity-onset character. The first sulfonylurea generation comprising compounds such as tolbutamide and chlorpropamide, is being gradually replaced by second generation sulfonylureas, such as glibenclamide and glipizide, which offer a markedly enhanced potency without a corresponding increase in toxicity.

The problem of glibenclamide determination has been overcome by employing chemical methods /1/, radioimmunoassay /2/, and gas chromatography /3/. Due to the low therapeutic dose of the

drug (2.5-15 mg/day) blood levels are also extremely low and create a growing need for specific analytical method of sufficient sensitivity. A recently developed radioimmunoassay is sensitive for the drug monitoring but because of cross-reactivity of the hydroxy-metabolites and individual variability lacks its specificity. GC requires a time-consuming derivatization step and the technique frequently lacks specificity since the intact compound and its metabolites may form identical derivatives. HPLC with fluorescence detection is based on pre-column derivatization, a time-consuming sample preparation, and the precision is not better than 9% /4/. HPLC with UV-detection represents a particularly sensitive technique for the determination of glibenclamide because of its high molar extinction coefficient at 228 nm /5/.

The HPLC method presented here is proposed for the routine monitoring of glibenclamide during the drug loading therapy because of its practicability, easy sample clean-up, using the solvent extraction procedure with salt precipitation of proteins /6/, no derivatization, good recoveries, good statistical evaluation and reliability. Injection of 400 samples of such prepared serum had no noticeable effect in pressure drop and column performance.

MATERIALS

Glibenclamide (1-{4-[2-(5-chloro-2-methoxybenzamido)ethyl]-phenylsulfonyl}-3-cyclohexylurea) [Gilemal® (Chinoin), Hungary; Euglucone® 5 (Boehringer-Hoechst), Austria], synthetic sample using p-hydroxybenzoate as the internal standard were used without further purification.

All chromatographic solvents were of HPLC grade. The inorganic reagents were prepared in distilled, deionized water. All reagents employed were analytical grade.

Mixtures of acetonitrile and phosphate buffer were used for elution. Degassing was carried out by sonication for 15 min.

APPARATUS

A Biotronik Model 3020 pump (Biotronik, Wissenschaftliche Geräte GmbH, Maintal, FRG), Model 504 Autosampler (Beckman Instruments G.m.b.H., Austria) loop injector fitted with a 20 µl loop were employed. A lichrosorb RP-18 250 x 4.6 mm I.D. column (particle size: 10 µm) (E. Merck, Darmstadt, FRG) was connected to a Biotronik 3030 UV-detector with variable wavelength set at 228 nm, 0.08 UFS and to a SP-4100 computing-integrator, 10mV, (Spectra-Physics GmbH, Darmstadt, FRG).

The mobile phase was 51:49 (v/v) 0.05 M sodium-dihydrogen-phosphate-acetonitrile pumped at a flow rate of 1.4 ml/min. The separations were carried out at ambient temperature.

STANDARD SOLUTIONS

A stock solution containing 1 mg of glibenclamide/ml was prepared by dissolving an accurately weighed sample of the reference standard in acetonitrile and stored at -15°C.

A stock solution containing 1 mg of the internal standard per ml was prepared by dissolving the appropriate quantity or the compound in acetonitrile and stored at -15°C.

On both occasions the stock solutions were diluted to the concentration of 10 ng/µl respectively.

PREPARATION OF CALIBRATION CURVE

Glibenclamide and the internal standard gave peaks under the described conditions with retention times of 9.64 and 9.03 min, respectively; see Fig. 1.

Calibration curves were prepared each day when samples were analyzed. 40 µl of diluted stock solution of the internal standard and 4.0, 8.0, 16.0, 32.0, 64.0, and 128.0 µl of the diluted glibenclamide stock solution were added to 1-1 ml of human serum. These standards were analyzed along with the samples (see Fig. 2).

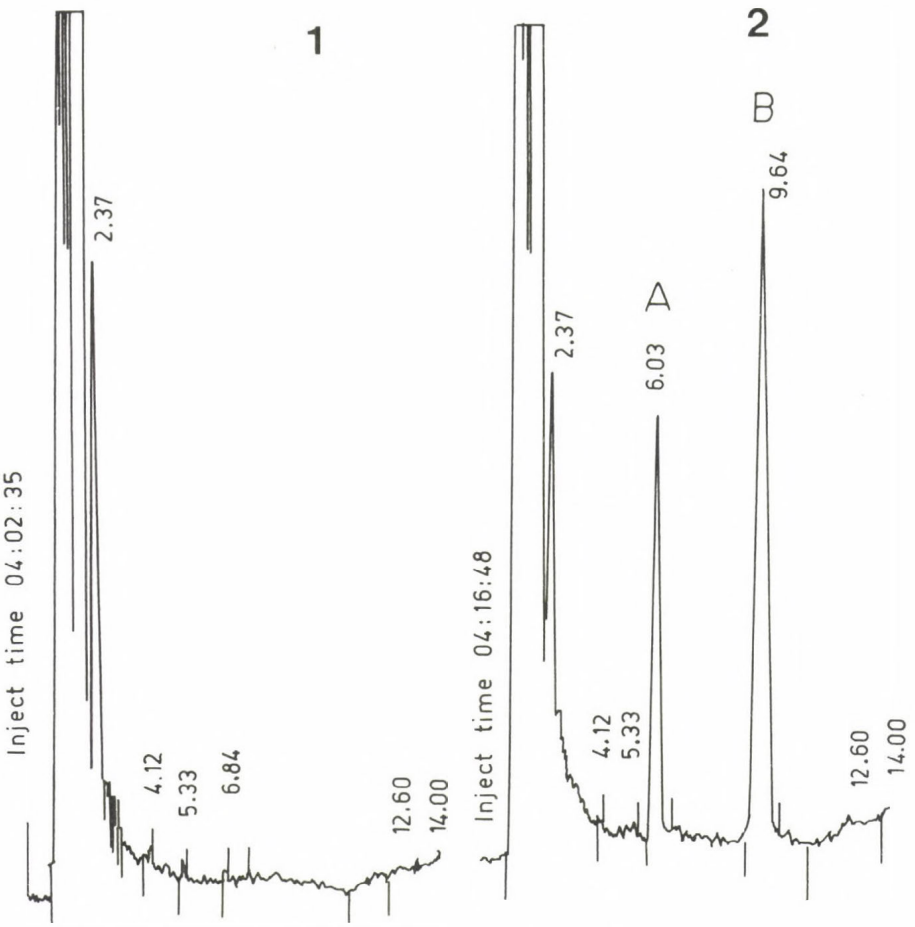

Fig. 1. Chromatograms of extracts from 1 ml serum.
1. Blank serum.
2. Serum spiked with glibenclamide and the internal standard

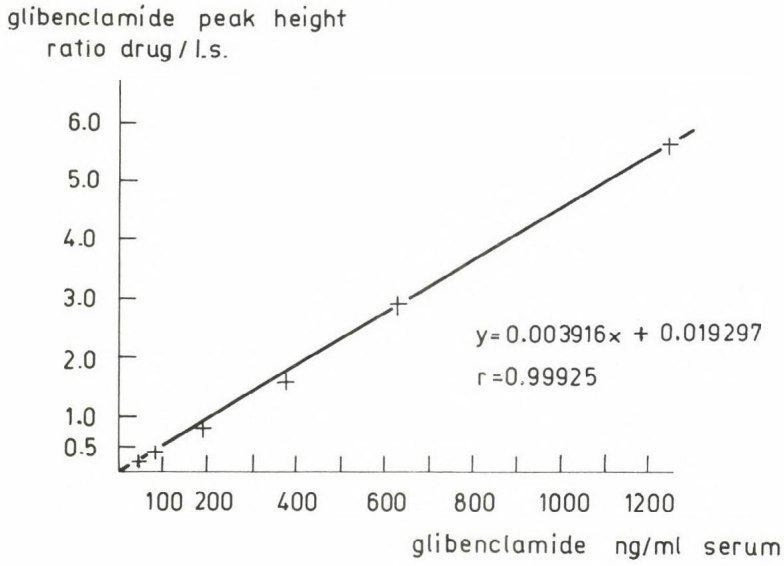

Fig. 2. Standard curve for the determination of glibenclamide in human serum, achieved by extraction of the drug from 1.0 ml serum containing 400 ng of internal standard

EXTRACTION OF SAMPLES

1 ml serum containing glibenclamide was measured into a 10 ml silanized centrifuge tube, then 40 µl of the internal standard was added. The sample was mixed for 20 sec. 0.1 ml of a 15% $ZnSO_4$ solution was added and shaken. For 5 min the samples were left to stand. 0.2 ml solution of $Ba(OH)_2$ was added, one more time mixed and incubated at room temperature for 5 min. Extraction was performed with 2 ml of ethyl acetate by shaking for one minute. Samples were centrifuged for 20 minutes at 3000 rpm, at 4°C. The ethyl acetate layer was transferred to another tube and evaporated to dryness under nitrogen stream. Residue was dissolved in 100 µl of the mobile phase and 20 µl were injected onto the column.

SERUM LEVEL STUDY

Eight healthy volunteers (selected according to the inclusion-exclusion criteria) were fasting overnight and received 10 mg of glibenclamide (two 5 mg tablets of Gilemal® or Euglucon®) with water (200 ml).

Each healthy volunteer remained under medical care for 24 hours.

For the determination of the serum concentration of the drugs blood samples were taken from the cubital veins, at 0, 0.5, 1, 2, 3, 4, 5, 6, 7, 9, 12, and 24 hours following each dose. The serum was removed and either immediately analyzed or stored at -20°C until just prior to analysis.

QUANTITATION

Calibration curves for glibenclamide were constructed by chromatographing spiked serum extracts and plotting the peak height ratios obtained for the drug vs. the internal standard against the concentration of the drugs. The glibenclamide concentration was calculated from the peak height ratio using the slope and intercept analysis of the calibration curves. The accuracy and precision of the assay is demonstrated in Table 1 giving results for the determination of glibenclamide in concentrations ranging from 40 to 1280 ng/mg of serum.

The calibration curve obtained was linear from 40 to 1280 ng/ml.

The absolute recovery of glibenclamide from serums containing 40, 80, 160, 320, 640, and 1280 ng/ml was determined by comparing the peak height obtained when 1 ml of serum residue and standards were chromatographed. The average recovery was 70 $\pm$ 5%. The glibenclamide detection limit was 10-20 ng/ml of serum extracted (S/N = 2), 0.08 AUFS. The average relative standard deviation was better than 3.01%.

Average serum profiles for 8 healthy volunteers, each dosed with 10 mg are shown in Fig. 3.

Table 1.

Precision Data by Internal Standard Method

	ng/sample added	ng/sample detected	%	N*	S.D.*	R.S.D.* %
1.	40	38.9±0.89	97.25	4	1.27	3.36
2.	80	85.88±1.9	107.35	4	2.15	3.25
3.	160	165.35±2.6	103.34	4	5.16	3.33
4.	320	315.06±5.1	98.45	4	10.11	3.05
5.	640	624.23±3.9	97.54	4	7.9	1.20
6.	1280	1287.74±21.5	100.60	4	43.04	3.39
average						3.01

*S.D. = standard deviation; R.S.D. = relative standard deviation; N = number of determinations

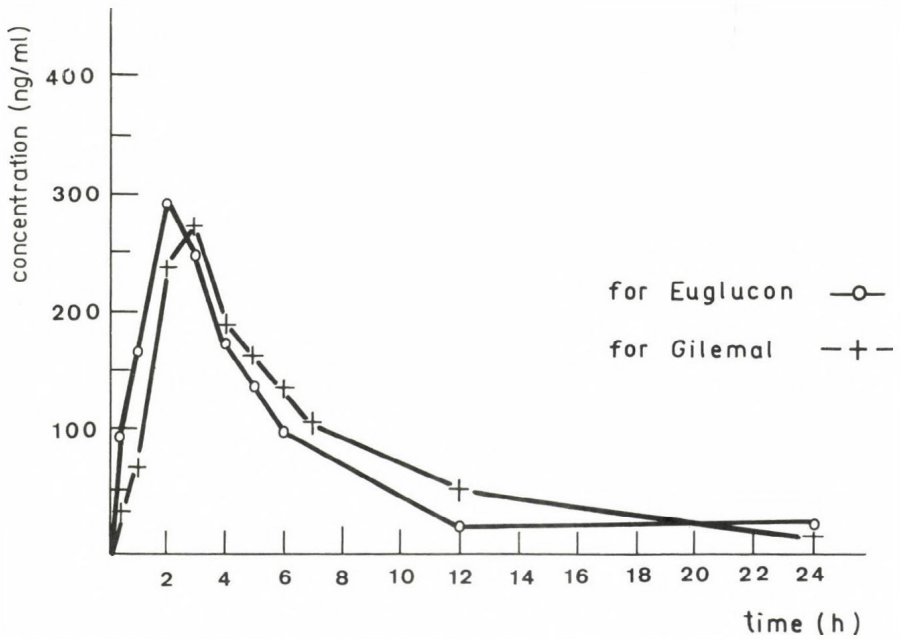

Fig. 3. Serum concentrations versus time profile for eight volunteers who received 2 x 5 mg glibenclamide with water before breakfast

Maximum serum concentration for 8 healthy volunteers are shown in Fig. 4.

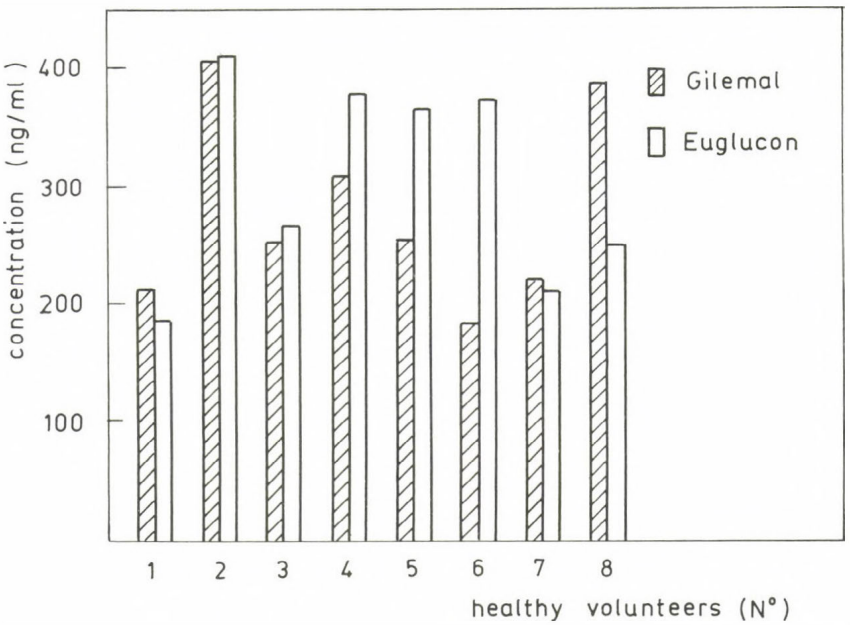

Fig. 4. Maximum serum concentration of Gilemal® and Euglucon® for 8 healthy volunteers

CONCLUSION

The presented method appears to be sufficiently selective, sensitive and rapid to allow accurate and precise measurements of serum concentrations of glibenclamide for routine determination of the drug.

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Chromatography '84
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SIMULTANEOUS HPLC-ANALYSIS OF PSYCHOTROPIC COMPOUNDS FROM VARIOUS CLASSES

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INTRODUCTION

In modern psychiatric clinic a great number of psychotropic preparations from various classes are used. Patients are exposed to the prolonged simultaneous pharmacotherapy with some medicines. There are, in the first place, neuroleptics of phenothiazine, thioxanthene and butyrophenone lines, tricyclic antidepressants of dibenzazepine and dibenzocycloheptene lines and anxiolytic preparations (tranquilizers) of benzodiazepine line (See Table I.).

All of these preparations have a significant effect on the central nervous system and are used for the appropriate clinical states in up-to-date clinics.

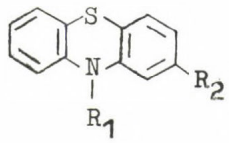
The high prevalence of these preparations is attributed to their action spectra. However, an effective employment of psychotropic medicines is impossible without extensive knowledge of their pharmacokinetics and pharmacodynamics. They are connected with the need to determine the concentrations of both preparations and their metabolites in the body of the patient or experimental animal (biological fluids: serum, urine, spinal fluid, etc.).

The routine pharmacy methods like photometry (colorimetry), UV- and IR-spectrophotometry, usual chemical methods, spectrofluorimetry, thin-layer and paper chromatography are used for the solution of this problem. However, recently they are more and more effectively substituted by high-performance liquid chromatography (HPLC).

Table I. Some widely used psychotropic preparations

Neuroleptics

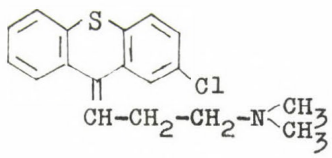
Phenothiazine line



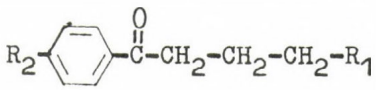
	$-R_1$	$-R_2$
Propazine	$-CH_2-CH_2-CH_2-N(CH_3)_2$	$-H$
Chlorpromazine	$-CH_2-CH_2-CH_2-N(CH_3)_2$	$-Cl$
Levomepromazine	$-CH_2-CH(CH_3)-CH_2-N(CH_3)_2$	$-OCH_3$
Perphenazine	$-CH_2-CH_2-CH_2-N\,N-CH_2-CH_2-OH$	$-Cl$
Trifluoperazine	$-CH_2-CH_2-CH_2-N\,N-CH_3$	$-CF_3$
Fluphenazine	$-CH_2-CH_2-CH_2-N\,N-CH_2-CH_2-OH$	$-CF_3$

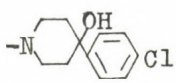
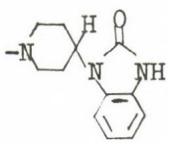
Thioxanthene line

Chlorprothixene



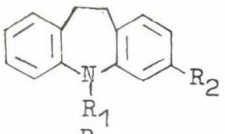
Butyrophenone line



	$-R_1$	$-R_2$
Haloperidol		$-F$
Benzperidol		$-F$

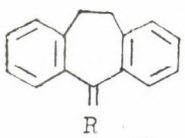
Tricyclic antidepressants

Dibenzazepine line

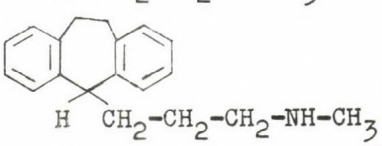


	$-R_1$	$-R_2$
Imipramine	$-CH_2-CH_2-CH_2-N(CH_3)_2$	-H
Chloroimipramine	$-CH_2-CH_2-CH_2-N(CH_3)_2$	-Cl
Trimipramine	$-CH_2-CH(CH_3)-CH_2-N(CH_3)_2$	-H
Desipramine	$-CH_2-CH_2-CH_2-NH-CH_3$	-H

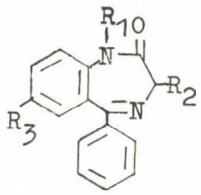
Dibenzocycloheptene line



=R

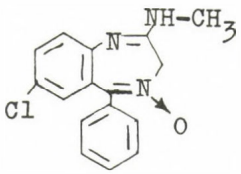
Amitriptyline	$=CH-CH_2-CH_2-N(CH_3)_2$
Nortriptyline	$=CH-CH_2-CH_2-NH-CH_3$
Protriptyline	

Tranquilizers
Benzodiazepine line



	$-R_1$	$-R_2$	$-R_3$
Diazepam	$-CH_3$	-H	-Cl
Oxazepam	-H	-OH	-Cl
Nitrazepam	-H	-H	$-NO_3$

Chlordiazepoxide



There is a considerable number of publications devoted to the HPLC-analysis of some psychotropic preparations and their metabolites |1-12|.

EXTRACTION PROCEDURE

One of the most important procedures in chromatographic analysis of biological and clinical compounds is an extraction procedure. The probability of the extraction of compounds from biological matters essentially depends on the extraction selectivity and the purification methods. Comparatively nonpolar solvents yield usually more clean chromatograms.

We have developed the psychotropic extraction procedure from serum (see Fig. 1), which is based on the different solubility of the compounds in organic and nonorganic phases having

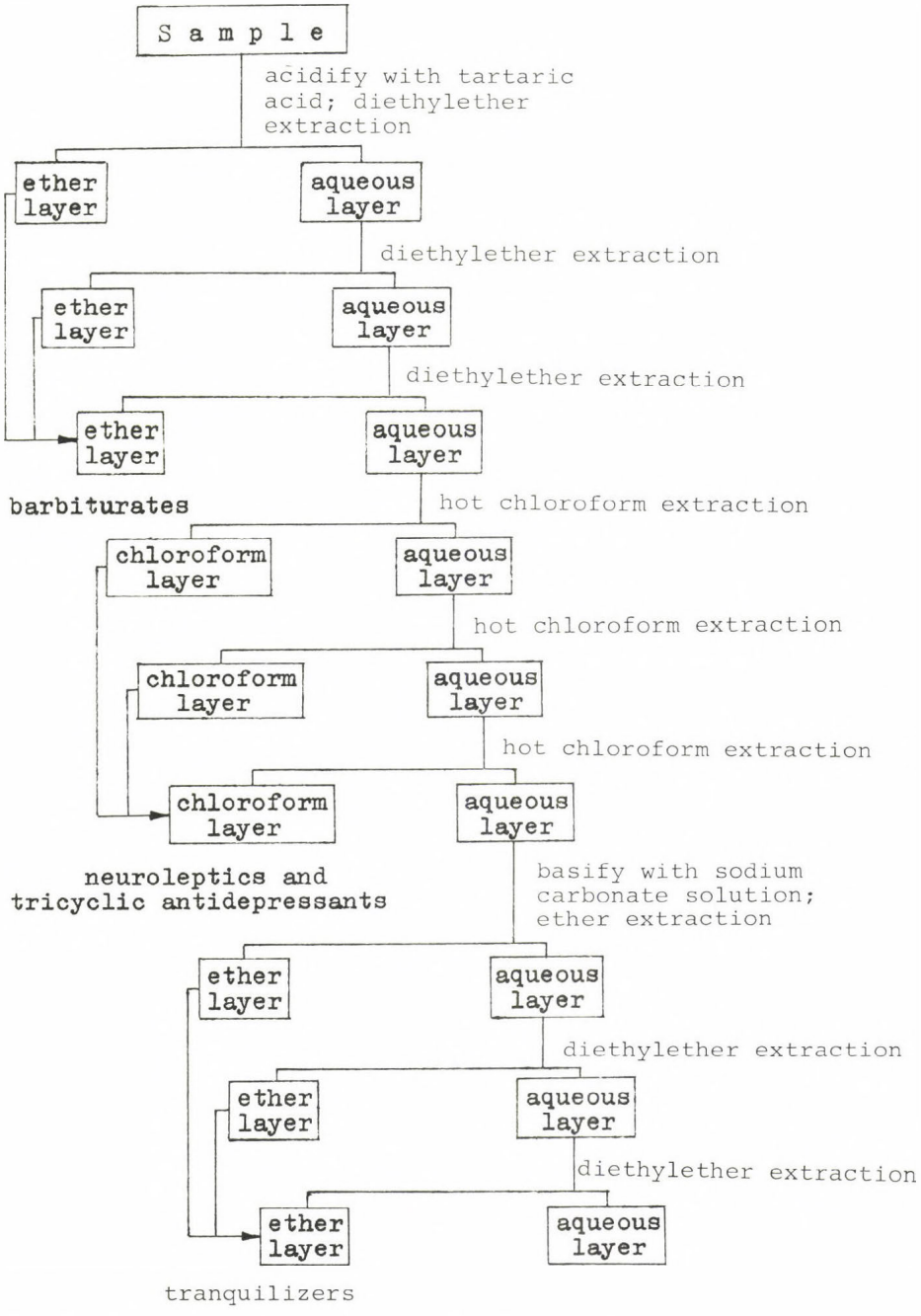


Fig. 1. Scheme of the extraction procedure

different pH values. Only nonionized compounds are soluble in organic solvents, thus only nonionized preparations are extracted at a certain pH value.

Extraction from aqueous solution occurs in accordance with the Nernst distribution equation. In this conection not only a single extraction with a large solvent portion is used, but repeated (3- or 4-fold) extractions, with small solvent volumes.

For the elimination of precipitation on the glassware walls all glassware have to be silanized prior to use with a 2% solution of dimethylchlorosilane in 1,1.1-trichloroethane.

HPLC-PROCEDURE AND RESULTS

Almost each of the preparations (with the exception of butyrophenones)indicated in Table I may be separated successfully on silica columns with HPLC. In our work the commercial liquid chromatograph "Milichrom" (USSR) was used with a stainless steel column packed with Silasorb 300, 5 μm, having approximately 2500 theoretical plates. UV-spectrophotometric detector was used at 254 nm for all preparations. When the maximum of the peak was reached, the chromatographic process may be stopped and the substance may be scanned from 190 to 360 nm.

Compounds of the butyrophenone line and their metabolites are separated on reversed phase columns with Silasorb C-18, 5 μm.

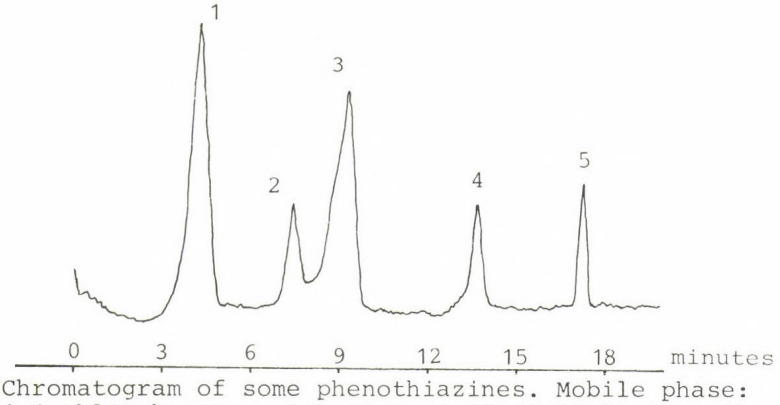


Fig. 2. Chromatogram of some phenothiazines. Mobile phase: 1:1 chlorobutane - isooctane + 1% diethylamine; flow rate: 1 ml/min; temperature; 25°C. Peaks: 1 - chlorpromazine; 2 - propazine; 3 - fluphenazine; 4 - levomepromazine; 5 - perphenazine

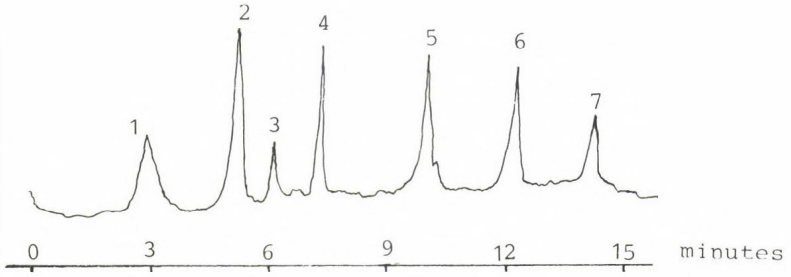


Fig. 3. Chromatogram of chlorpromazine and some of its metabolites from patient serum. Mobile phase: - 33:65:2 acetonitrile - trimethylpropane - 2-aminopropane; flow rate: 1 ml/min; temperature: 22°C. Peaks: 1,3,5,6-methylized chlorpromazine metabolites; 2 - chlorpromazine; 4,7 - chlorpromazine sulfoxides

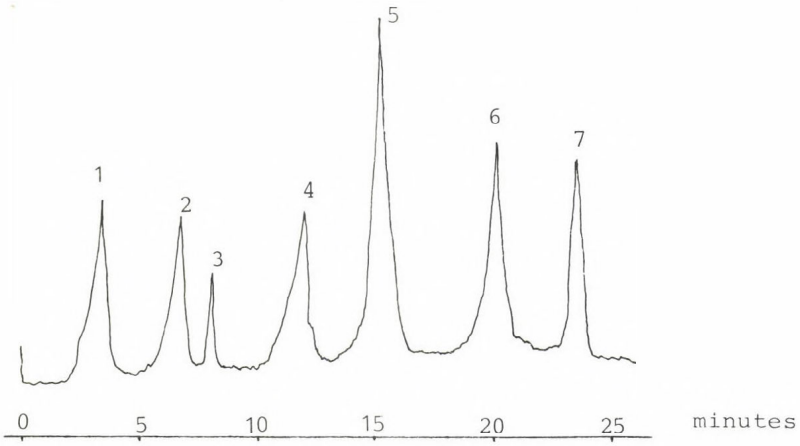


Fig. 4. Chromatogram of some tricyclic antidepressants. Mobile phase: 99:1 methanol - aqueous ammonium hydroxide: flow rate: 2 ml/min; temperature: 23°C. Peaks: 1 - trimipramine; 2 - chloroimipramine; 3 - amitriptyline; 4 - imipramine; 5 - nortriptyline; 6 - desipramine; 7 - propriptyline

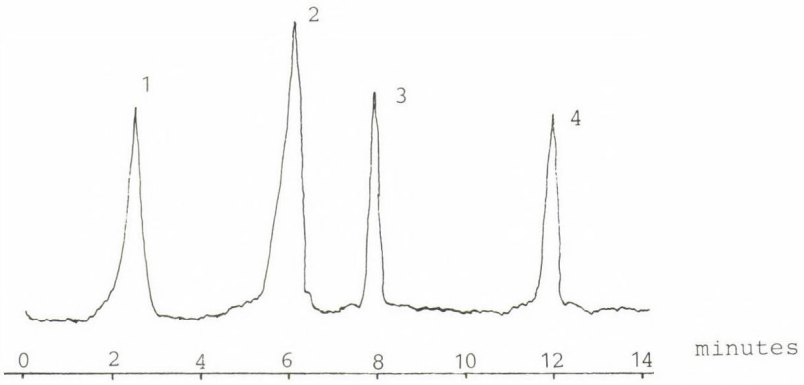


Fig. 5. Chromatogram of some tranquilizers. Mobile phase: 80:18:2 - n-heptane - isopropanol - methanol; flow rate: 1 ml/min; temperature: 25°C. Peaks: 1 - oxazepam; 2 - chlordiazepoxide; 3 - nitrazepam; 4 - diazepam

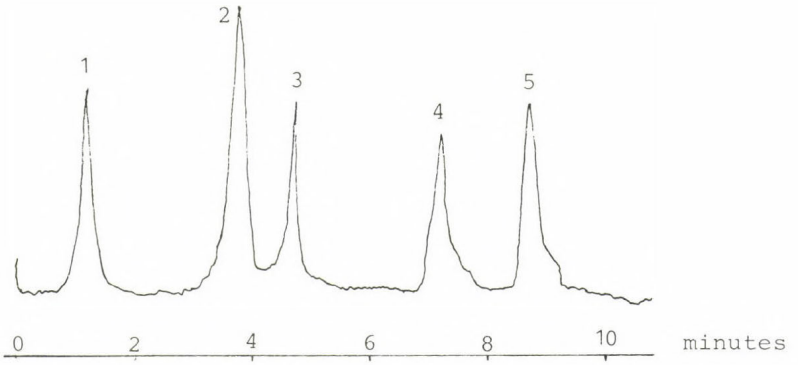


Fig. 6. Chromatogram of haloperidol and some of its metabolites from patient serum. Mobile phase: 65:33:2 - methanol - 0.01 M KCl - glacial acetic acid; flow rate: 2 ml/min; temperature: 22°C. Peaks: 1 - haloperidol; 2,3,4,5 - various haloperidol metabolites

The described extraction procedure makes it possible to preconcentrate the psychotropic preparations before the HPLC analysis by evaporating under vacuum.

Figs 2-5 show a few typical chromatograms of psychotropic preparations.

CONCLUSIONS

The described procedures may be successfully used in the pharmacokinetic research of psychotropic preparations. Unknown metabolites have to be isolated preparatively and analyzed with UV- or IR spectroscopy, NMR spectroscopy and mass spectrometry. The extraction procedure may be extended for urine, spinal fluid, etc. These methods are used successfully for the rapid analysis in toxicologic investigations.

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DETERMINATION OF PORPHYRIN ISOMERS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Reversed-phase high-performance liquid chromatography with fluorescence detection is useful in the determination of urinary porphyrin carboxylic acid isomers. Copro I and III - as well as uro I and III porphyrin isomers are simultaneously separated.

INTRODUCTION

The determination of porphyrins in urine plays a major role in the diagnosis and classification of the porphyrias. Extraction methods still used are able to distinguish the uroporphyrin, coproporphyrin and protoporphyrin /1/. Physiologic porphyrins can be separated on silica gel layers as their methyl esters /2/. Recently the technique of high-performance liquid chromatography has been applied to porphyrin analysis in biologic samples /3-5/. The separation and determination of coproporphyrin I and III isomers is clinically important. It has been used for the differential diagnosis of the porphyrias and/or distinguishing the congenital erythropoietic porphyria (CP III much more CP I, much UP) and the Dubin - Johnson syndrome from the Rotor syndrome /6-8/. Uroporphyrinogen III is the precursor of haem and vitamin B_{12}. In the absence of porphobilinogen deaminase, preuroporphyrinogen is spontaneously rearranged into uroporphyrinogen I. The separation methods for uroporphyrin I and III isomers in urine were important for the

diagnosis of congenital porphyria (UP I much more UP III), and for developing an assay for uroporphyrinogen III cosynthetase where the quantitative separation of the I and III isomers is essential /9/. The porphyrin isomers were determined by paper and thin-layer chromatography /10, 11/, spectrofluorimetrically detected ion-exchange chromatography system /12, 13/ and high-performance liquid chromatography. Uroporphyrin I and III octamethyl esters have been separated on a microporasil column /14, 15/. Coproporphyrin I and III tetramethyl esters have been separated for diagnosis of hepatic porphyrias /16/. The uro- and coproporphyrin I and III free acid isomers were determined by reversed-phase HPLC on ODS - Hypersil /2, 8, 12/.

We describe here an effective method for the simultaneous separation of all copro- and uroporphyrin isomers and for the isolation of porphyrin free acids using reversed-phase chromatography. Effective separation of the porphyrins is achieved by gradient elution.

EXPERIMENTAL

Reagents

Coproporphyrin I and III tetramethyl esters were obtained from Sigma (St. Louis, Mo, USA). Uroporphyrin ester according to Waldenstrom was purchased from Sigma London Ltd. (Poole, UK.). All porphyrin esters were hydrolyzed overnight in 6 N HCl, and diluted with distilled water until a concentration of 3 N HCl was obtained. The concentrations of porphyrins were calculated from diluted standard preparations and the molecular extinctions /18/.

Acetonitrile was purchased from Reanal (Budapest, Hungary); all other reagents were analytical grade.

HPLC apparatus and mobile phases

Liquochrom-307 (Labor MIM, Hungary) liquid chromatograph was used with a Hitachi MPF-4 fluorescence spectrophotometer detector. The excitation and emission wavelengths were 406 nm

and 619 nm, respectively. OE-320 Labor MIM injector fitted with 20 µl sample loop was used for injection. The separation was carried out on a column (25 cm x 4 mm) packed with Chromsil (10 µm spherical silica, chemically bonded with octadecylsilyl groups, Labor MIM, Hungary).

The solvents for gradient elution were acetonitrile (15 v/v) in 1.0 M ammonium acetate buffer (pH = 5.15)(solvent A) and acetonitrile (50 v/v) in 1.0 M ammonium acetate buffer (pH = = 5.15) (solvent B). The buffer was prepared in the following way: 30 ml 96% acetic acid was added to 1 litre of 1.0 M ammonium acetate solution. Solvent A was made according to recent findings /9/ on the separation of uroporphyrin I and III. Solvent B was made as demonstrated by Wright et al. /8/ for the separation of coproporphyrin I and III. We used these two solvents for the determination of all porphyrin isomers in gradient elution. The column was equilibrated with distilled water before the sample was injected. The solvents were mixed in the course of gradient elution. The flow rate was maintained at 48 ml/hour throughout.

Sample preparation

A rapid procedure has been adapted in which an anion-exchange resin is used to concentrate total porphyrins from urine /13/. Varion AT - 660 (Dowex 1 type) anion-exchange resin (chloride form, Nitrokémia, Hungary) was used without pretreatment. For column preparation, an appropriate amount of the resin is suspended in distilled water and poured into the column (K 16/20, Labor MIM-Hungary) to give a 1.6 x 2.0 cm resin bed. After excess water has flowed through, the column is ready for use. A 10 ml aliquot of the urine is then pipetted into the reservoir of the column and allowed to flow through the anion-exchange resin. Unchanged materials are washed through the column with three 10-ml portions of distilled water. These washes are discarded. Porphyrins are eluted from the resin by adding four 10-ml portions of 3 N HCl. The eluent solutions are combined and evaporated to dryness in vacuum and dark. The dry porphyrins are dissolved in 0.2 ml of 3 N HCl.

A 0.02-ml aliquot of the dissolved porphyrins is then injected in the HPLC column.

RESULTS AND DISCUSSION

Table 1 shows the separation parameters of a standard porphyrin mixture using the present HPLC system.

Table 1. Capacity ratios(k'), separation factor(α) and relative separation factors(R_s) for porphyrins on reversed-phase ODS-silica packings

Substance	k'	α	R_s
Uroporphyrin I	5.3		
Uroporphyrin III	5.8	1.1	0.6
Heptacarboxylic porphyrin	8.4	1.4	2.8
Coproporphyrin I	15.9	1.9	8.9
Coproporphyrin III	16.9	1.1	0.9

Eluent for gradient elution: solvent A, 15 v/v acetonitrile in 1.0 M ammonium acetate buffer(pH = 5.15); solvent B, 50 v/v acetonitrile in 1.0 M ammonium acetate puffer(pH = 5.15). Column, Chromsil(10 µm); flow rate 48 ml/hour.

The clinically important porphyrins, including coproporpyrin I and III, and Waldenstrom-type uroporphyrin (mixture of uroporphyrin I and III, and heptacarboxylic porphyrin) were satisfactorily separated. This is comparable to or better than other systems described for the separation of porphyrin free acids. The isomers were eluted in the following order: uroporphyrin I and III, heptacarboxylic porphyrin and coproporphyrin I and III. An important feature of the present system is the ease with which retention and resolution can be precisely controlled by manipulation of pH, buffer concentration and organic modifier content in the mobile phase.

Table 2. Urinary porphyrins in clinically confirmed porphyria

Patient	Concentrations (µg/l)					Porphyrin diagnosis*
	Uro-I	Uro-III	Hepta	Copro-I	Copro-III	
A (N.F.)	1339	627	1053	84	149	PCT
B (S.I.)	653	435	473	21	44	PCT
C (N.S.)	655	523	262	21	34	PCT
D (N.L.)	802	475	897	38	161	PCT
E (P.S.)	342	228	628	34	91	PCT
F (K.I.)	806	704	954	29	102	PCT
G (F.B.)	562	441	482	72	241	PCT
H (M.J.)	870	353	54	22	49	AIP
I (T.H.)	118	95	71	238	1425	VP
J (P.J.)	489	145	158	405	1099	CEP
K (C.A.)	12	8	-	25	48	normal

*PCT = porphyria cutanea tarda; AIP = acute intermittent porphyria; VP = varigate porphyria; CEP = congenital erythropoetic porphyria.

We have also analyzed several cases of clinically diagnosed porphyria, and values for these are summarized in Table 2.

Most normal urine samples contain only coproporphyrin and perhaps a small amount of uroporphyrin. Normal urine contains about 31 $\pm$ 15 µg/day CP I and about 72 $\pm$ 27 µg/day CP III /17/.

Porphyria cutanea tarda (PCT) is characterized by high levels of uroporphyrin and heptacarboxyl porphyrin. In PCT, the uroporphyrin-coproporphyrin and heptacarboxyl porphyrin-coproporphyrin ratios are greater than one, and these ratios can be used to recognize various subclinical states of hepatic porphyria /19/.

The single urine specimen that was from a patient with acute intermittent porphyria(AIP) also conformed to previously reported patterns, showing highly elevated uroporphyrin, but not heptacarboxylic porphyrin /20, 21/.

Specimen from one patient with varigate porphyria(VP) contained highly elevated coproporphyrin as the dominant component in the porphyrin pattern /21/.

The diagnosis for one patient was congenital erythropoetic porphyria(CEP). In this case the CP III> CP I as the dominant components and much UP were highly elevated /8/.

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EXTRACTION AND HPLC ANALYSIS OF CANNABINOIDS

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SUMMARY

Different solvents were compared and evaluated for the extraction of cannabinoids from plant materials and resins. Clean-up and enrichment of the extracted cannabinoids was investigated using adsorbents of different polarities.

The major cannabinoids of forensic interest were separated by HPLC on reversed-phase columns using different eluent systems. Resolution of Δ^9-THC and Δ^8-THC was also demonstrated by using reference standards.

Identification of the separated components were identified by using reference standards as well as by the investigation of the HPLC fractions using GC-MS and FT-IR techniques.

Quantification of the active components was investigated by measuring the peak heights and the peak areas of the compounds.

On the basis of these investigations a method was elaborated for the separation, identification and quantification of active cannabinoids in materials of forensic interest.

INTRODUCTION

For detailed examination and identification of the cannabinoids a large number of extraction, chromatographic and spectroscopic techniques have been investigated.

The major constituents to be found in Cannabis plant materials and Cannabis products associated with the pharmacol-

ogical effects of the drug are Δ^9-tetrahydrocannabinol (Δ^9-THC), cannabidiol (CBD) and cannabinol (CBN) the chief active compound being Δ^9-THC. The Δ^8- compound (Δ^8-THC) has also been found but most of the publications refer to them collectively as THC /1/.

Many common organic solvents are very effective at extracting the cannabinoids both from the plant and from the various cannabis preparations. Light petroleum, chloroform, ethanol, methanol-chloroform mixture, hexane and methanol were investigated as solvents and the stability of the resulting extracts were also studied /2-6/.

There are some contradictions in the literature as regards extraction efficiency, co-extraction of non-cannabinoid compounds and stability of the extracts in the different solvents investigated. It is surprising that solvents of such different characters and polarities have been found satisfactory for extracting cannabinoids.

The cannabinoids extracted can then be separated chromatographically by several techniques.

Cannabis constituents have been traditionally assayed using TLC for qualitative studies and GLC for quantitative work. There are several TLC systems that can be used for the separation of some of the main cannabinoids by using a number of specific spray reagents as visualising agents, most of all Fast Blue B /7-10/.

Some of the cannabinoids can be separated and subsequently quantified using gas-liquid chromatography (GLC) /1-6, 11-12/. The use of GLC, however, causes thermal degradation of cannabinoid acids to the corresponding cannabinoids resulting in the loss of valuable information. Various derivatization techniques have been described to overcome this problem, but none are completely satisfactory.

The most specific and sensitive separation systems are based on combined gas chromatography and mass spectrometry (GC-MS). Capillary columns have recently been employed for cannabinoid separations, with excellent results. Best results are obtained after derivatization, usually as trimethylsilyl (TMS) ethers /1, 13-15/. Although sensitive and capable of yielding much

information, methods based on GC-MS require complex and expensive equipment.

Recently some papers have been published of the application of high-performance liquid chromatography (HPLC) to the analysis of Cannabis products. The HPLC analysis of cannabinoids overcomes many of the disadvantages inherent in other methods. The separation is effected at room temperature and in the absence of air, which precludes thermal degradation, isomerization and oxidation reactions. HPLC using both normal and reversed-phase techniques has been successfully applied to cannabinoid analysis /1, 15, 16-23/. In early works UV detection at 254 nm was carried out but it was established that detection at 220 nm significantly improved the sensitivity for the main compounds /22/.

Although several methods have been suggested for the determination of cannabinoids in various Cannabis products there are still some questions open regarding the efficiency of extraction, clean-up of co-extracted non-cannabinoids, resolution, identification and quantification of the major constituents.

The objective of the present study was to develop a complex but relatively simple method starting from the extraction of various Cannabis products which would enable unambigous determination of the cannabinoid content of samples of different origins.

EXPERIMENTAL

Samples

Cannabis plant materials (Cannabis Sativa) and Cannabis resins seized by the Hungarian Customs Service were investigated.

Extraction

Extraction of samples was performed in ultrasonic bath. 500 mg plant material and 50 mg Cannabis product, respectively, was extracted 3 times with 10 ml of the solvent. Successive

extractions from plant materials and resins used in this study showed that the cannabinoids were exhaustively extracted within 10 minutes. The combined extracts were filtered through Whatman GF/D glass-wool filter then evaporated to dryness over water bath at 60 ^{o}C under N_2. The dry residue was taken up in 1-10 ml solvent for further processing.

In order to compare the performance of different solvents, methylene chloride, tetrahydrofuran, methanol and n-hexane were investigated.

Clean-up

In order to separate the co-extracted non-cannabinoids various adsorbents of different polarities were investigated. Comparative experiments were carried out in a small glass microadsorber using the technique developed for the enrichment and clean-up polyaromatic hydrocarbons (PAH) in environmental samples /24/.

The adsorbents used were as follows;
Carbon Molecular Sieve, Phase CMS, 170-200 mesh
Amberlit XAD-2 polymer(purified) (Applied Science), 20-60 mesh
Lichroprep RP-18 reversed-phase packing (Merck), 25-40 µm
Silicagel (Woelm), 30-60 µm

For the development of the analytical technique Silica and C-18 SEP-PAK cartridges of Waters Associates were used.

Clean-up was carried out by using an aliquot of the solutions obtained in the extraction step. The adsorbent was wetted with the appropriate(weakest) solvent then the sample was applied on the adsorbent. The sample was fractionated by elution with small amount (5-10 ml) of solvents of different polarities. In order to establish optimum fractionation small fractions (2-5 ml) of the eluates were collected and analysed by HPLC and TLC.

HPLC apparatus and conditions

For the preliminary investigations of the extraction efficiency and the clean-up procedure a Labor MIM Model Liquochrom

2010 high-performance liquid chromatograph, including a reciprocating piston pump, an injection valve equipped with a 20 µl sample loop, a stainless-steel column (250 x 4.6 mm I.D.) packed with 10 µm particles of Chromsil C-18 reversed-phase packing (Labor MIM) and a variable wavelength ultraviolet detector (Liquodet Model 308) was used. For the development of the analytical procedure a Hewlett-Packard Model 1084B high-performance liquid chromatograph with variable wavelength UV detector, equipped with a Nucleosil-10 C-18 reversed-phase column (250 x 4.6 mm I.D.) was used.

70:30, 75:25, 80:20 methanol-water and 75:5:20 methanol-tetrahydrofuran-water eluent systems were investigated as the mobile phase. All separations were carried out isocratically, at ambient temperature.

For identification of the separated components retention data measured with reference standards were used. Quantitation of the major cannabinoids was performed by constructing calibration curves with reference standards, by measuring both peak heights and peak areas.

Gas chromatography-mass spectrometry (GC-MS)

For positive identification of the major cannabinoids and in order to control peak homogenity GC-MS measurements were carried out using the peak fractions collected from several injections (5-10) on the HPLC column. The combined peak fractions were evaporated under N_2 in water bath, the residue was taken up in 50 µl hexane and 1 µl was injected into the GC column.

A Hewlett-Packard Model 5985B GC-MS instrument was used, equipped with a packed column (2 m x 3 mm I.D.) containing 3% OV-17 on 100-120 mesh Chromosorb W support and using He as the carrier gas (30 ml/min). Column temperature was 250 ^{o}C, injector and detector were held at 280 ^{o}C.

Fourrier-Transform Infrared spectrometer

The structure of the THC compound identified by GC-MS was confirmed by FT-IR analysis using a DIGILAB FTS-14A instrument.

Reagents

Solvents used in extraction, clean-up and preliminary investigations were of analytical grade (Reanal, Budapest) and were distilled in glass before application.

The solvents used with the HP Model 1084B were of Lichrosolve (Merck) quality. Some reference standards (Δ^8-THC, Δ^9-THC, CBN, CBD) were received from the United Nations, Division of Narcotic Drugs, Vienna, as standard solutions. Some other standards of lower purity were obtained from other sources and were used as received.

RESULTS AND DISCUSSION

HPLC separation of cannabinoids

In order to determine the efficiency of extraction and clean-up procedures HPLC separation of the major natural constituents of Cannabis sativa on reversed-phase column was accomplished using reference standards.

Retention times were determined by injecting standard solutions of the compounds. Average of three successive injections were taken as the retention time. Capacity factors were calculated relative to the retention time of the solvent peak (methanol, t_o = 0.91 min). Relative retentions to Δ^9-THC were also calculated.

Retention data for cannabinoid standards on Nucleosil-10 C-18 reversed-phase column with 80-20 methanol-water eluent system using 2 ml/min flow rate are given in Table 1. The separation of a model mixture is shown in Fig. 1. Table 1. and Fig. 1. show that fairly good resolution can be obtained between the critical cannabinoid pairs, namely between CBD and CBG, as well as between Δ^9-THC and Δ^8-THC. The time of analysis is about 9 minutes which is reasonably short for routine analysis.

Experiments carried out with weaker eluent systems (70:30 and 75:25 methanol-water) resulted in much longer analysis time (37 and 23 min, respectively) with only a small increase in

Table 1.

Retention of cannabinoid standards on Nucleosil-10 C-18 reversed-phase column with 80:20 methanol-water eluent system

Peak number	Compound	Abbreviation	Retention time, min	Relative retention*	Capacity factor, k'	Selectivity α
1	Cannabidiol	CBD	3.83	0.60	3.21	1.15
2	Cannabigerol	CBG	4.27	0.67	3.69	1.37
3	Cannabinol	CBN	5.50	0.86	5.04	
4	Δ^9-Tetrahydrocannabinol	Δ^9-THC	6.38	1.00	6.01	1.19
5	Δ^8-Tetrahydrocannabinol	Δ^8-THC	6.76	1.06	6.43	1.07
6	Cannabichromen	CBC	8.26	1.29	8.08	1.26

*Relative to Δ^9-THC

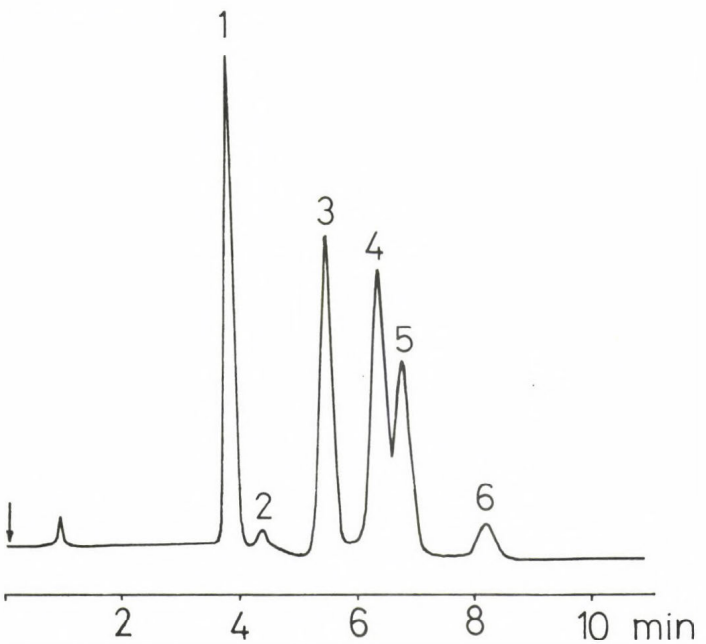

Fig. 1. Separation of cannabinoid standards
Column: Nucleosil-10 C-18 reversed-phase. Eluent: 80:20 methanol-water, 2 ml /min.Detection: UV 220 nm. Compounds: see Table 1.

Table 2.

Retention of cannabinoid standards on Nucleosil-10 C-18 reversed-phase column with 75:5:20 methanol-THF-water eluent system

Peak number	Compound	Retention time, min	Relative retention	Capacity factor, k'	Selectivity α
1	CBD	3.79	0.65	3.16	1.17
2	CBG	4.29	0.73	3.71	1.25
3	CBN	5.13	0.88	4.64	1.17
4	Δ^9-THC	5.85	1.00	5.43	1.09
5	Δ^8-THC	6.28	1.07	5.90	1.21
6	CBC	7.40	1.27	7.13	

*Relative to Δ^9-THC

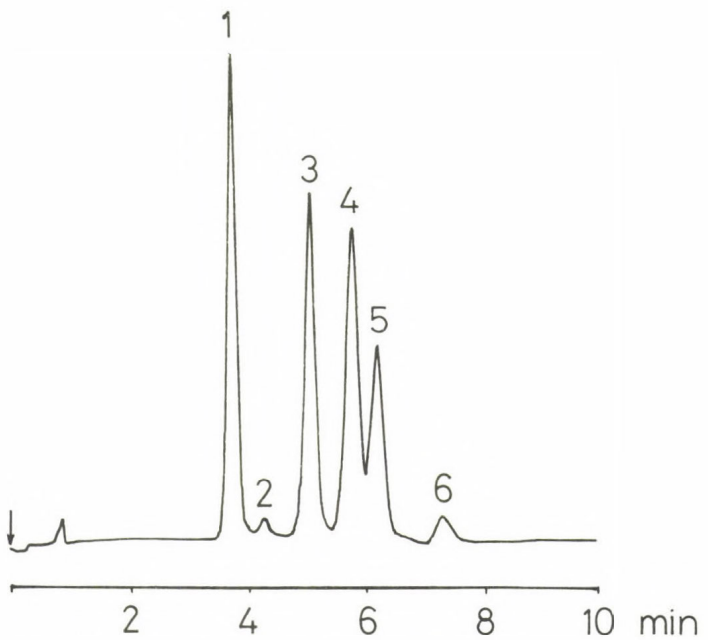

Fig. 2. Separation of cannabinoid standards
Column: see Fig. 1. Eluent: 75:5:20 methanol-THF-water

resolution. For this reason the 80:20 methanol-water system was used for further investigations.

Needless to say, the same separation can be carried out in much shorter time using the new high-speed columns with 5-10 cm length and 3 μm particles, however, at the time of our investigations such columns were not available.

In a later phase of the work a modified eluent system was tested in order to increase the selectivity by using 75:5:20 methanol-THF-water eluent system. Retention data measured for the cannabinoid standards are given in Table 2. The separation of a model mixture is shown in Fig. 2. It can be seen that the retention times are shorter and the selectivities are somewhat higher than that obtained with the 80:20 methanol-water system. Nevertheless, it must be added that the resolution of Δ^9-THC and Δ^8-THC was of lesser concern because Δ^8-THC was not found in significant amounts in preliminary studies of different samples.

Extraction

Extraction of a cannabis plant material and a cannabis resin was performed under identical conditions with the four solvents given above. The colours of the various extracts were different indicating the differences in the extraction of dye-stuffs present in the samples. In Table 3, the colours of the different extracts obtained are given.

Table 3.
Colours of the various extracts of cannabinoids

Solvent	Sample	Colour
Methylene chloride	C.plant	green-brown
	C.resin	deep yellow
Tetrahydrofuran	C.plant	green
	C.resin	yellow
Methanol	C.plant	green-yellow
	C.resin	yellow
n-Hexane	C.plant	light yellow
	C.resin	light yellow

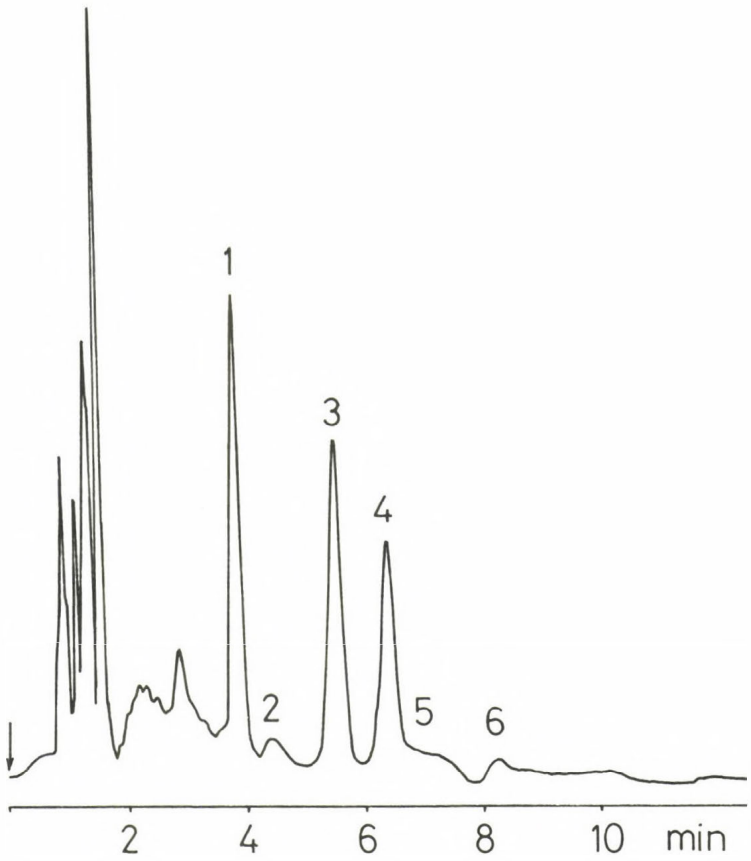

Fig. 3. Chromatogram of a Cannabis resin extract by methanol extraction
Operating conditions: see Fig. 1.

Extraction was carried out as described, and the dry residue was taken up in 1 ml methanol for HPLC analysis. In the chromatograms peak heights of the major cannabinoids were measured and evaluated. From these preliminary investigations it was concluded that the efficiency of extraction of cannabinoids was lowest with methylene chloride, followed by tetrahydrofuran. Methanol and n-hexane resulted in practically identical efficiency for cannabinoids. In Fig. 3. the chromatogram of a resin extract obtained with methanol, while in Fig. 4. the chroma-

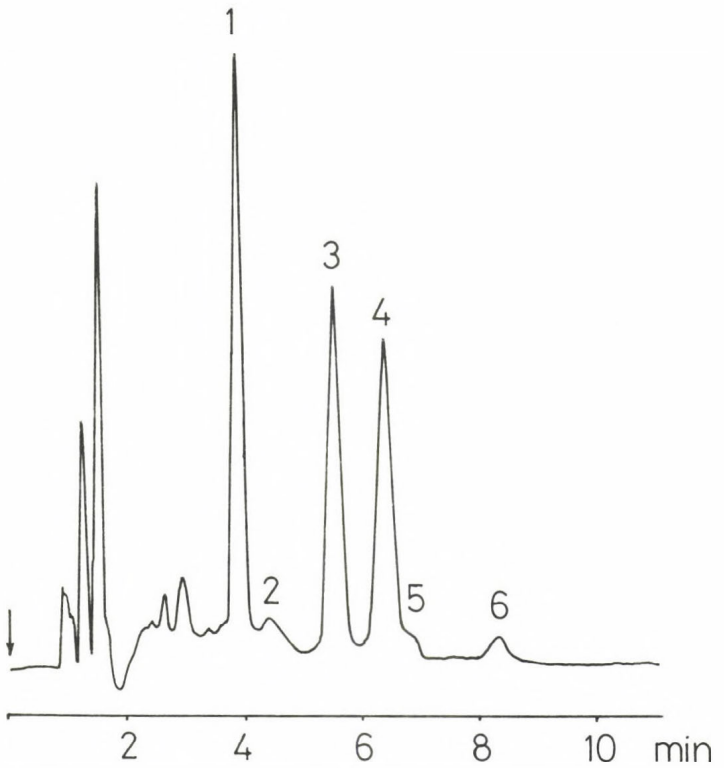

Fig. 4. Chromatogram of a Cannabis resin extract by n-hexane extraction. Operating conditions: see Fig. 1.

togram of the n-hexane extract are shown. It can be seen in the figures that the methanol extract contains a larger amount of highly polar non-cannabinoid components, among them coloured substances, in accordance with the stronger colour of the extract. For that reason n-hexane was chosen as the preferred extracting agent and further investigations were conducted with the n-hexane extracts of the samples.

Clean-up

Experiments carried out with the four different adsorbents investigated have shown that no reasonable separation of non-cannabinoids from cannabinoids was achieved with the active carbon and XAD-2 adsorbents.

Investigations performed with C-18 reversed-phase packing and silica, however, have shown that with appropriately selected solvent program the non-cannabinoids can be separated from the cannabinoids of interest.

In order to develop a routine procedure for the clean-up of the extracts detailed investigations were carried out with silica and C-18 SEP-PAK cartridges.

Clean-up on silica SEP-PAK cartridge:

The silica cartridge was wetted with 5 ml n-hexane, then 5 ml of the n-hexane extract was introduced. The cannabinoids were eluted with 10 ml benzene. The benzene fraction was evaporated to dryness, the residue was taken up in 1 ml methanol, filtered, then analysed by HPLC. Fig. 5. shows the chromatogram of the benzene fraction. It can be seen that most of the non-canna-

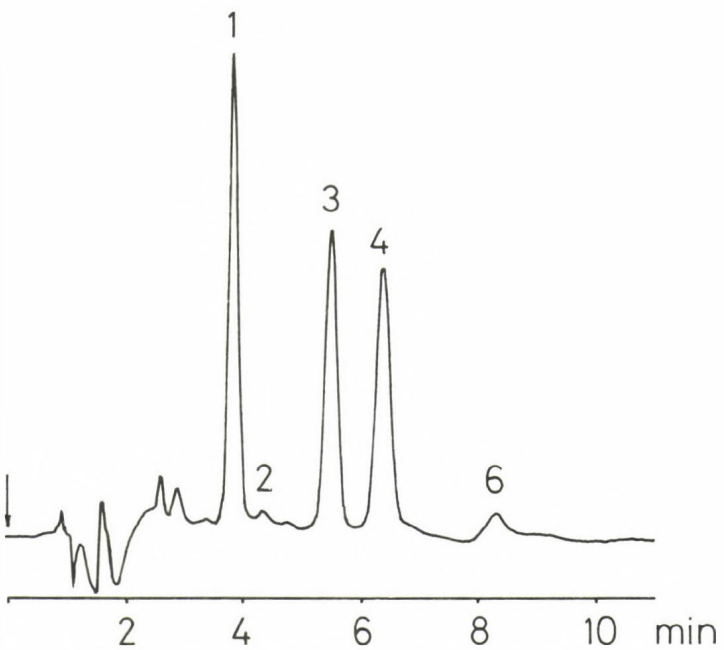

Fig. 5. Chromatogram of a Cannabis resin extract after clean-up on silica
Operating conditions: see Fig. 1.

binoids were retained on the silica adsorbent. The silica cartridge was regenerated by eluting with 10 ml methanol, then dried with N_2 gas. The regenerated cartridge can be used at least 4-5 times for new samples without loss of activity depending on the amount of coloured matters in the sample.

Clean-up on C18 SEP-PAK cartridge:

The C-18 cartridge was wetted by 5 ml methanol followed by 5 ml distilled water.

The hexane extract was evaporated to dryness, the residue was taken up in 1 ml methanol and the methanol solution was introduced into the cartridge. The cannabinoids were eluted by 10 ml of 85:15 methanol-water solvent. This fraction was evaporated to dryness, the residue taken up in 1 ml methanol, filtered, and then analysed by HPLC.

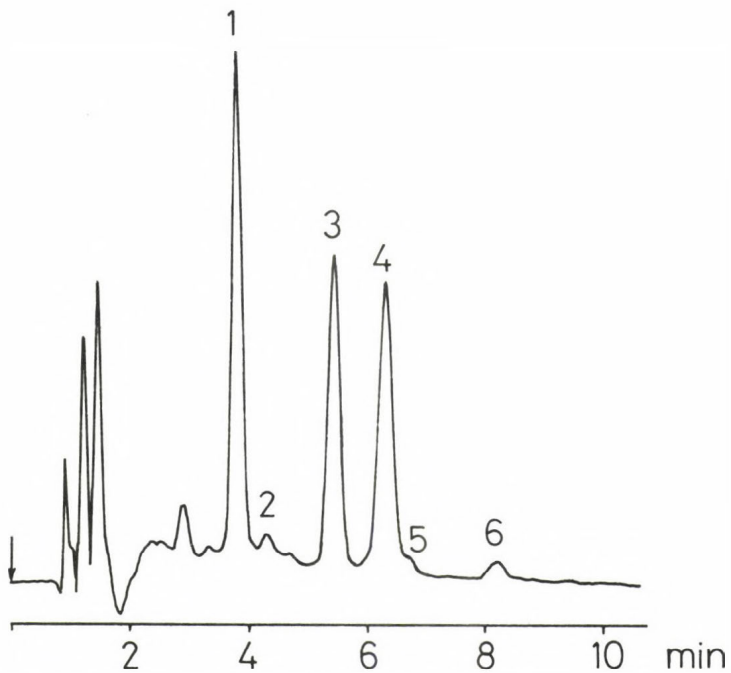

Fig. 6. Chromatogram of a Cannabis resin extract after clean-up on C-18 reversed-phase packing
Operating conditions: see Fig. 1.

Fig. 6. shows the cannabinoid fraction. In comparison to Fig. 5. it can be seen that some of the highly polar non-cannabinoids are only partly removed.

The C-18 cartridge was regenerated by elution with 10 ml methanol followed by 10 ml methylene chloride, then again by 10 ml methanol. The regenerated cartridge can be used 10-15 times for new sample preparation.

Clean-up on silica and C-18 SEP-PAK cartridges:

In order to exclude the interference of both polar and non-polar compounds co-extracted with the cannabinoids a two-step clean-up procedure was developed using silica and C-18 adsorbents in series.

The first step is identical with the procedure described for silica cartridge. 0.5 ml of the methanol solution obtained

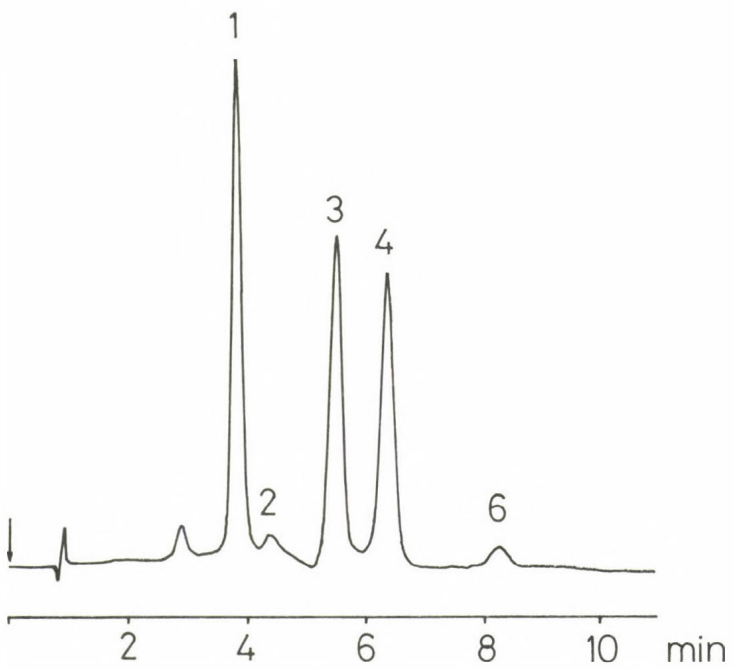

Fig. 7. Chromatogram of a Cannabis resin extract after two-step clean-up
Operating conditions: see Fig. 1.

after the evaporation of the benzene fraction was introduced into the C-18 cartridge. The second step is identical with the procedure described for C-18 cartridge.

Fig. 7. shows the chromatogram of the cannabinoid fraction obtained with the two-step clean-up procedure. There is not much difference to be seen in comparison with the chromatogram obtained with clean-up on silica cartridge but the possibility of interference of co-extracted non-cannabinoids is highly reduced because of the application of a polar and a non-polar adsorbent.

Recovery in clean-up procedure

Efficiency of the two-step clean-up procedure was determined by the introduction of a known amount of Δ^8-THC standard.

100 µl of a standard Δ^8-THC solution in ethanol (5 mg/ml) was evaporated to dryness under standard conditions given above and the residue was taken up in 10 ml n-hexane. 5 ml n-hexane solution was applied into a silica cartridge and then the first clean-up step, followed by the second step was carried out as described above. The residue was taken up in 1500 µl methanol.

As reference standard, an aliquot of the standard Δ^8-THC solution was evaporated and taken up in 1500 µl methanol.

The two samples were chromatographed and the peak areas determined. Comparison of the amounts obtained demonstrated that the recovery of Δ^8-THC after the clean-up procedure was 95%. In the chromatogram of the prepared sample other peaks than Δ^8-THC peak could not be observed indicating that no degradation took place during the clean-up procedure.

Quantitative analysis

Calibration curves were constructed for the major cannabinoids using sample injection of standard solutions of different concentrations. Measurements were carried out with UV detection at 254 and 220 nm. In accordance with literature data considerably higher sensitivity was achieved at 220 nm. Calibration was established at 220 nm. Fig. 8. shows the cali-

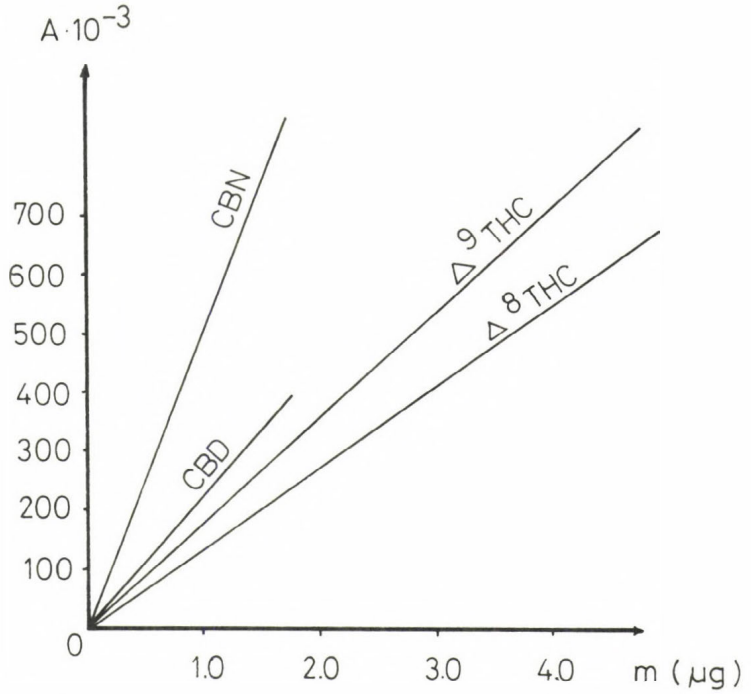

Fig. 8. Calibration curves for some cannabinoid standards (A = peak area).

CBD	a=217984	b= 2769	r=0.999
CBN	a=488471	b=11304	r=0.999
Δ^9-THC	a=178018	b=11285	r=0.998
Δ^9-THC	a=137643	b=-1293	r=0.995

bration curves prepared by peak area measurement for Δ^9-THC, Δ^8-THC, CBN and CBD. Δ^8-THC contains a weaker chromophore than that of Δ^9-THC demonstrated by the calibration curves (smaller slope).

It can be seen from the figure that peak areas give linear relationships in the concentration range investigated. Linear relationships were also established by peak height measurements. From both types of calibration curves it can be concluded that the detection limit for Δ^9-THC under the conditions investigated is about 50 ng.

From the chromatogram of a Cannabis resin seized by the Customs Service, shown in Fig. 9. quantification of the major

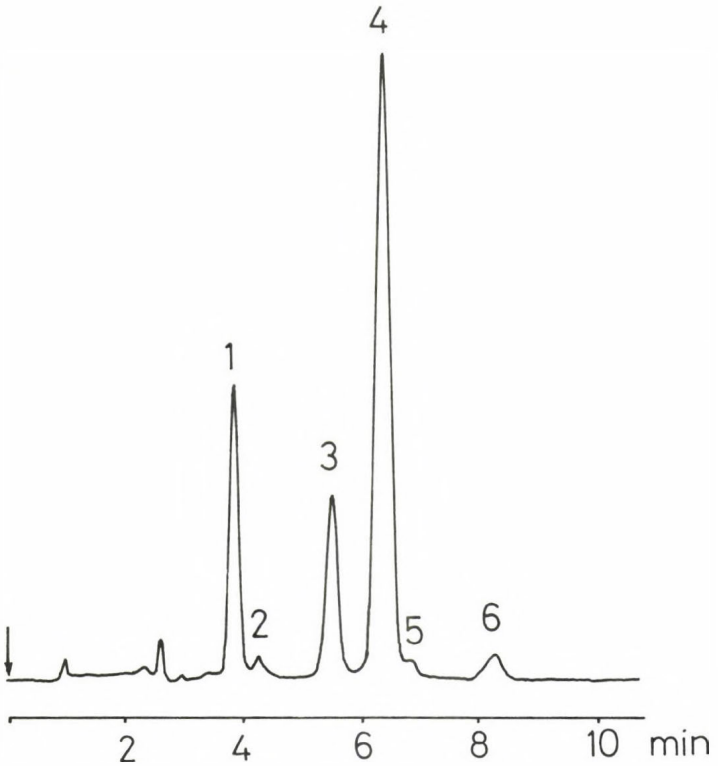

Fig. 9. Chromatogram of a Cannabis resin extract for quantitative evaluation
Operating conditions: see Fig. 1.

cannabinoids was carried out using the calibration curves demonstrated in Fig. 8. Calculated to the weighed resin the sample contained 0.91% CBD, 0.40% CBN and 3.55% Δ^9-THC. In Fig. 9. a small shoulder can be observed in the tail of the Δ^9-THC peak corresponding to the Δ^8-THC isomer. In accordance with the results obtained for different samples and literature data Δ^8-THC was not found in significant amounts in various Cannabis plants and products.

As a comparison a Cannabis plant sample (500 mg) prepared and processed in the same way contained 0.65% CBD, 0.025% CBN and 0.74% Δ^9-THC.

Confirmative identification of Δ^9-THC

Δ^9-THC is regarded as the major psychoactive component of Cannabis products. The compound found by chromatographic separation as Δ^9-THC was confirmed by GC-MS and FT-IR investigations.

From the Cannabis resin sample after the clean-up procedure (Fig. 9.) 5 injections were performed into the HPLC column and the fractions containing Δ^9-THC were collected. The combined peak fractions were evaporated to dryness, the residue was taken up in 50 µl n-hexane and 1 µl was injected into the GC column. The mass spectrum of the compound was registered and matched to library spectrum /25/. It was established that the compound is THC and no contaminants could have been detected.

The same sample was also investigated by FT-IR spectrometer. The IR spectrum obtained was matched to the reference spectrum of the UN /26/ and it was unambigously established that the compound is identical to Δ^9-THC.

CONCLUSIONS

For the extraction of the main neutral cannabinoids from plant materials and Cannabis products n-hexane is the preferred solvent: the extraction can be performed in ultrasonic bath with good efficiency.

In order to improve life time of HPLC columns and to avoid possible interferences with coextracted non-cannabinoids a clean-up procedure is proposed prior to HPLC analysis. The two-step clean-up procedure developed performs adsorption on silica and C-18 reversed-phase packing ensuring high recovery of the major cannabinoids and eliminating interferences of both polar and non-polar non-cannabinoids.

HPLC on C-18 reversed-phase column with methanol-water eluent system is adequate for the resolution and determination of the major cannabinoids in samples of Cannabis plant materials and Cannabis products after clean-up of the sample extracts.

Positive identification of chromatographically separated THC by GC-MS and FT-IR techniques demonstrated the reliability of chromatographic analysis and homogenity of the separated peaks.

On the basis of calibration curves prepared with reference standards ng quantities of cannabinoids can be detected and reliable quantitation can be carried out.

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Chromatography '84
H. Kalász and L.S. Ettre (Eds)

APPLICATION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY TO PHARMACEUTICAL ANALYSIS

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EGYT Pharmacochemical Works, Budapest, Hungary

SUMMARY

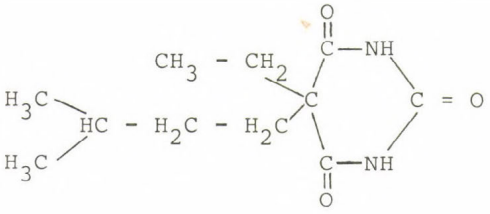
HPLC determination of amobarbital, glutethimide and promethazine hydrochloride content of Tardyl tablets is described. Rapid determination of paracetamol and carisoprodol content of Scutamil C coated tablets is given by HPLC.

I. A SIMPLE ASSAY METHOD FOR THE DETERMINATION OF AMOBARBITAL, GLUTETHIMIDE AND PROMETHAZINE HYDROCHLORIDE CONTENT OF TARDYL TABLETS

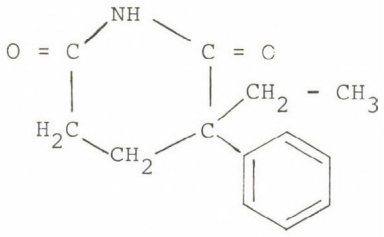
The control of active ingredients of multicomponent tablets usually demands a lot of time. It means a special work to control the products of mass production where, in the case of small ingredient, the examination of uniformity of the dosage unit is also necessary.

Tardyl tablets are one of the important products of EGYT Pharmacochemical Works, which contain 125 - 125 mg of amobarbital (I), glutethimide (II) and 7.5 mg of promethazine hydrochloride (III) (see Fig. 1 for the structures).

Previously quantitative control of the active ingredients was carried out by the determination of their light absorption in different agents. It needed much time. Instead of this method we developed a simple, fast and reliable reversed-phase HPLC method, which is suitable for the simultaneous determination of all three active ingredients.



I.



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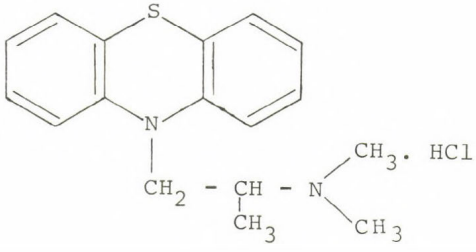


III.

Fig. 1. Structures of the active ingredients of Tardyl tablets. I = amobarbital; II = glutethimide; III = promethazine hydrochloride

EXPERIMENTAL

Reagents

All chemicals utilized were analytical-reagent quality and were used without further purification. HPLC-grade acetonitrile (Merck, Darmstadt, FRG) was used as the mobile phase. Water was deionized and doubly distilled in glass.

Apparatus

A PYE UNICAM (Cambridge, England) Model LC 3 liquid chromatograph equipped with a Model 7125 Rheodyne sample injection valve, a Model LC-XPD pump, a Model PU 4020 variable wavelength UV detector and a Hewlett Packard (Avondale, Pennsylvania, USA)

Model 3390 A integrator-recorder was used in our work. The analytical column was PARTISIL 10 ODS (Whatman, Clifton, NJ, USA), 250 x 5 mm I.D., particle size 10 µm.

HPLC conditions

Potassium phosphate buffer (0.067 M, pH 3.2 adjusted by dropwise addition of diluted phosphoric acid) was filtered, then degassed in an ultrasonic bath. Acetonitrile was filtered and degassed in the same way prior to mixing with the potassium phosphate buffer. An isocratic mobile phase system of 45:55 (v/v) acetonitrile - potassium phosphate buffer was delivered with a flow rate of 1.2 ml/min.

The other operating conditions were as follows; measured inlet pressure 4.7 MPa (47 bar); column temperature 30°C; sensitivity 0.08 a.u.f.s. Quantitation was based on peak area measurement.

Calibration curves

Standard solution of amobarbital, glutethimide and promethazine hydrochlorid was prepared at concentrations between 10 g/ml and 20 g/ml of amobarbital and glutethimide and 0.8 g/ml and 1 g/ml of promethazine hydrochloride in the eluent. A 20 µl volume of standard solution was injected in triplicate and the peak areas were averaged to prepare the calibration graphs for each component.

Assay of pharmaceutical formulation

Twenty tablets were accurately weighed and finely powdered. 0.36 g tablet powder was weighed accurately into a 100-ml volumetric flask and dissolved in 70 ml of the eluent in an ultrasonic bath (15 min.). The solution was allowed to equilibrate at ambient temperature, then the volume was completed to mark with the eluent. The solution was filtered through dry filter paper, discarding the first 10 ml of the filtrate. 0.3 ml filtrate was diluted to 25 ml with the eluent.

20 µl of the sample solution was chromatographed under the operating conditions described above.

Quantitative calculations were performed by the external standard method.

Results and discussion

Fig. 2 shows a typical chromatogram of a standard solution. Amobarbital, glutethimide and promethazine hydrochloride have the capacity factors of 2.35, 2.87 and 4.80 respectively.

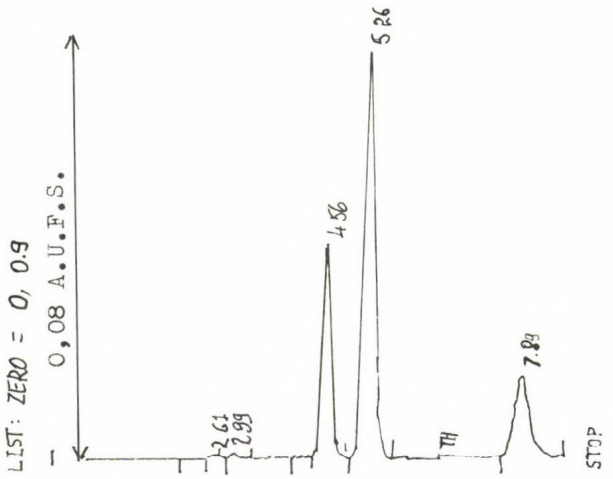


Fig. 2. HPLC separation of amobarbital (15 g/ml), glutethimide (15 g/ml) and promethazine hydrochloride (0.9 g/ml). Conditions: column, Partisil 10 ODS; mobilephase, 45:55 acetonitrile-potassium phosphate buffer (0.067 M pH 3.5); detector, 210 nm; flow rate, 1.2 ml/min. Retention times: amobarbital 4.56 min; glutethimide 5.26 min.; promethazine hydrochloride 7.89 min

The analysis time was 15 min. The former UV method required 1.5-2 hours. As all of the three active ingredients can be analyzed in one determination, the method is suitable for production control and for checking the uniformity of the dosage units of tablets.

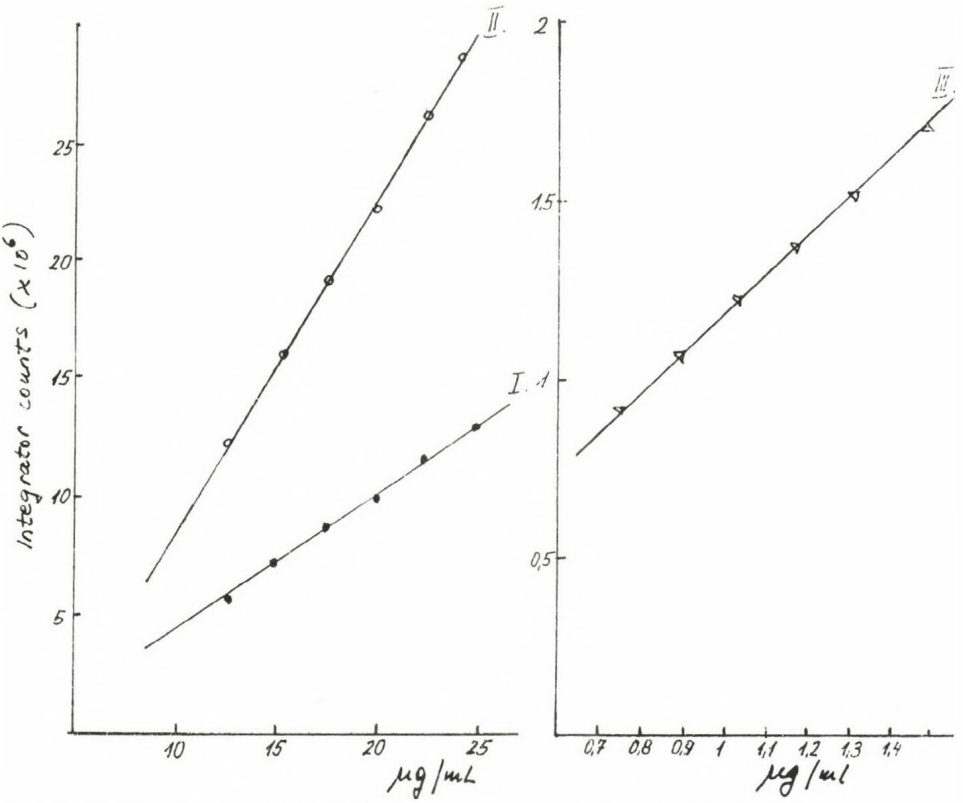


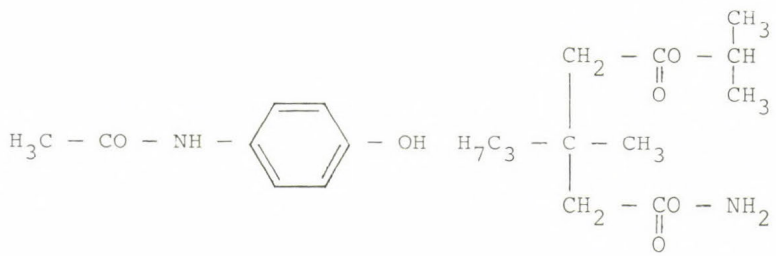
Fig. 3. Calibration plots for amobarbital (I), glutethimide (II) and promethazine hydrochloride (III). Each point represents the average of at least three determinations

The reproducibility and accuracy of the method were very statisfactory. The relative standard deviations of the HPLC method (based on ten measurements) were the following:

amobarbital	0.5%
glutethimide	0.6%
promethazine hydrochloride	0.63%

II. RAPID DETERMINATION OF THE PARACETAMOL AND THE CARISOPRODOL CONTENT OF COATED SCUTAMIL C TABLETS

Coated Scutamil C tablets are widely used as an antirheumatic agent. The active ingredients of the tablets are paracetamol (I) and carisoprodol (II)(Fig. 4).



I. II.

Fig. 4. Structures of paracetamol (I) and of carisoprodol (II)

The determination of paracetamol was previously carried out by light absorption measurement at 249 nm. The carisoprodol content could only be determined by indirect measurement. As the coated Scutamil C tablets are placed on the market in great quantities, this complicated method occupies a considerable manpower in the laboratory.

The reason why a simple method could not be used for the determination of carisoprodol is that paracetamol always interferes. The only way was to separate the two conponents. Although carisoprodol does not have a chromophor group a significant UV response is obtained at 200 nm which is suitable for its quantitative determination.

EXPERIMENTAL

Reagents

All chemicals utilized were analytical-ragent quality. HPLC-grade acetonitrile (Merck, Darmstadt, FRG) was used as the mobile phase. Water was deionized and twice distilled from glass.

Apparatus

We used a PYE UNICAM (Cambridge, England) Model LC 3 liquid chromatograph which consists of a Model LC-XPD pump, a Model PU 4020 variable wavelength UV detector, a Model LC-XP system controller programmer, and a Rheodyne Model 7125 sample injection valve. A Hewlett-Packard (Avondale, Pennsylvania, USA) Model 3390 A integrator-recorder was used.

The column was Partisil 10 ODS (Whatman, Clifton, NJ, USA), 250 x 5 mm I.D., particle size 10 µm, jacketed and held at constant temperature (30°C) by means of a water-circulating bath.

HPLC conditions

Mixture of acetonitrile and 0.067 M potassium phosphate buffer pH 3.2 were used as the mobile phase. The potassium phosphate buffer was adjusted to pH 3.2 with dilute phosphoric acid and degassed under vacuum and by ultrasonic bath immediately before use. Chromatographic runs were performed isocratically, using 40:60 (v/v) acetonitrile potassium phosphate buffer, with a flow rate of 1.4 ml/min. The measured inlet pressure was 3.7 MPa (37 bar).

Other conditions

Column temperature 30°C; injection volume 20 µl; detector wavelength 200 nm: detector sensitivity 0.08 a.u.f.s. Quantitation was based on peak area measurement.

Calibration curves

Standard solution:

About 100 mg of paracetamol standard and 150 mg of carisoprodol standard were weighed into a 50 ml volumetric flask and dissolved in the eluent. 0.475 ml, 0.5 ml and 0.525 ml aliquots of this solution were diluted to 10 ml with the eluent. 20 µl aliquots of each solution were chromatographed at least in triplicate.

Sample solution:

Twenty coated tablets were accurately weighed and finely powdered. 0.32 g of the powdered tablet mass was weighed into a 50 ml volumetric flask and dissolved in 70 ml of the eluent, in an ultrasonic bath (15 min). After equilibration at ambient temperature the volume was made up with the eluent. The solution was filtered through a dry filter paper, discarding the first 10 ml of the filtrate. 0.5 ml filtrate was diluted to 10 ml with the eluent. 20 µl of the sample solution was injected into the liquid chromatograph.

Quantitations were performed by the external standard method.

RESULTS

A typical separation of paracetamol and carisoprodol by reserved-phase HPLC is shown in Fig. 5.

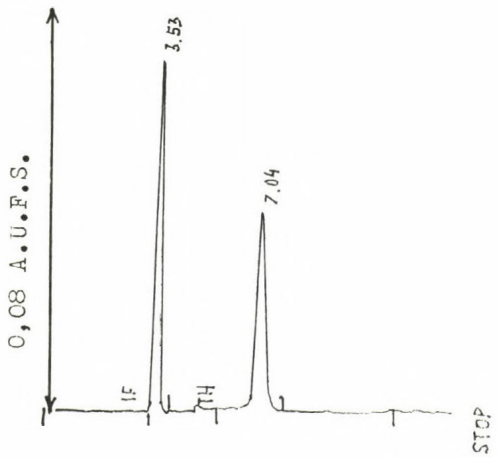


Fig. 5. HPLC separation of paracetamol (0.1 mg/ml) and carisoprodol (0.15 mg/ml). Conditions; column, Partisil 10 ODS; mobilephase, 40:60 acetonitrile - 0.067 M potassium phosphate buffer (pH 3.2); detector, 200 nm; flow rate, 1.4 ml/min. Retention times: paracetamol 353 min; carisoprodol 7.04 min

The described HPLC assay method for Scutamil C is simple, fast and reliable. Relative standard deviation values were (based on ten measurements):

paracetamol	0.3%
carisoprodol	0.6% .

Akadémiai Kiadó
Budapest, 1986

Chromatography '84
H. Kalász and L.S. Ettre (Eds)

HPLC ANALYSIS OF ECDYSTEROIDS OF SILENE OTITES (L) Wib.

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SUMMARY

Ecdysteroids originated from the extract of Silene otites (L) Wib. have been analysed by HPLC on C-18 reversed phase column. Possibility of screening as well as of purity control of the isolated substance is discussed.

INTRODUCTION

Ecdysteroids are a particular group within the class of steroids having a highly hydroxylated steroid skeleton with the following characteristics: a 6-keto-7-ene moiety in the B-ring, a cis-fused A/B ring junction, 14-alpha oriented hydroxyl and a complete sterol side chain in position 17 (Fig. 1).

Due to their important role in the development of insects, there is a considerable interest in pure ecdysteroids as biochemicals for research purposes. Significant attention has been focused on the biosynthesis, metabolism and receptor-hormone interactions of the ecdysteroids in insects. The possibility of using insect moulting steroids for the genetic control of insects or perhaps as chemotherapeutic agents against parasites have generated studies worldwide on these steroid hormones/1/.Sources with high ecdysteroid content and also containing special ecdysteroid composition are needed. Similarly, there is an effort

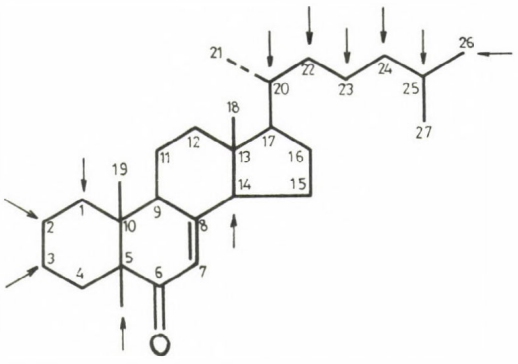


Fig. 1. The general structure of ecdysteroids. Arrows indicate the positions of possible substitutions

to find hitherto unknown members of the group or ecdysteroids exerting antiecdysone effect.

The main sources for the production of ecdysteroids are various plant raw materials since the ecdysteroid content of some plants is essentially higher (up to 1%) than that of the insects. The qualitative and preparative separation of ecdysteroids can be performed by using various chromatographic techniques. For preparative purposes, column liquid chromatography and droplet countercurrent chromatography (DCCC) have been used /2-5/ while the analytical differentiation and quantitation of ecdysteroids have been done mainly using paper chromatography, thin-layer chromatography (TLC), gas chromatography, radio-immunoassay and various physico-chemical methods /6-10/. In the course of our previous work /11/ several plant species which are native in the southern part of Hungary were found as adequate sources for the production of ecdysteroids. For preparative purposes, column chromatography on alumina, DCCC, precipitation and fractionated crystallisation were used /12/, while the screening of the plant material and the monitoring of the successive isolation were mainly done by TLC.

At a later stage Silene otites (L) Wib. (Caryophyllaceae) was found to be one of the best sources of ecdysteroids, and the analytical procedure was completed by the use of high-performance liquid chromatography (HPLC) for both screening of

the extracts before further purification, and the purity control of the isolation of pure preparations. While TLC permits the simultaneous investigation of a large number of spots and the use of specific colour reagents, HPLC provides a high separation efficiency in a short time, and the ultraviolet absorbence of ecdysteroid compounds permits their sensitive detection. Therefore, HPLC may be the proper method for the analysis of small amounts of ecdysteroids.

MATERIALS AND METHODS

The five ecdysteroid compounds listed in Fig. 2 were isolated from Silene otites (L) Wib. as previously described /13/. Silene otites (L) Wib. was harvested in the vicinity of Szeged (Hungary). The whole fresh plants were collected, dried, milled and extracted with methanol (10 ml methanol per g of the plant). The extract was filtered and evaporated to dryness under reduced pressure. The residue was dissolved in a small amount of methanol and subjected to analysis. The ecdysteroid stock solutions contained each steroid in a concentration of 2 mg/L. The solutions were stored at -40^{o}C; at this temperature no decomposition could be observed.

Methanol, triethylamine and phosphoric acid (all three of analytical grade) were purchased from Reanal (Budapest, Hungary); 1-butanesulfonic acid sodium salt monohydrate (98%) was obtained from Aldrich Chemical Company (Milwaukee, WI USA). The stationary phase for HPLC was Chromsil C-18, 6 µm and represented the kind gift of Dr. Ohmacht (Medical University, Pécs, Hungary). 250 x 4.2 mm columns were used in our work.

Two solutions were used as the mobile phase: they contained a triethylamine - phosphate buffer (10 mL triethylamine, 35 mL phosphoric acid and 90 mg 1-butanesulfonic acid sodium salt monohydrate per litre aqueous solution) and methanol, in different concentrations:

solution No. 1.: 40:60 methanol - triethylamine-phosphate buffer,

solution No. 2.: 50:50 methanol - triethylamine-phosphate buffer.

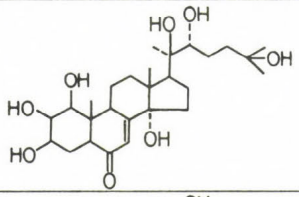
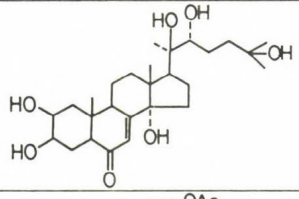
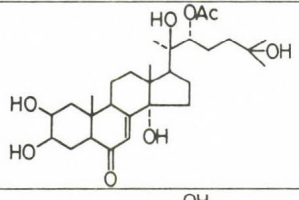
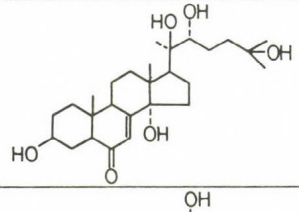
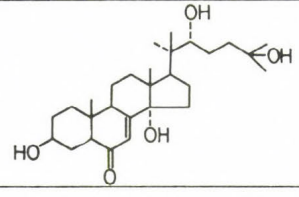
No.	Structure	Chemical name Common name
1		1,20 - Dihydroxyecdysone Integristerone A
2		20 - Hydroxyecdysone ß - Ecdysone
3		20 - Hydroxyecdysone - 22 - acetate
4		2 - Deoxy - 20 - hydroxyecdysone 2 - Deoxy - ß - ecdysone
5		2 - Deoxyecdysone 2 - Deoxy - α - ecdysone

Fig. 2. The structure of edcysteroids studied in this paper. The serial numbers indicate their elution in Figs 3-6

Liquochrom 2010 liquid chromatograph of Labor MIM (Budapest, Hungary) was used in our work. It consisted of an OE-312 Liquopump solvent delivery system, an injector with 20 µl loop, and a OE-306 UV detector. The chromatograms were recorded on an OH 814/1 Recorder (Radelkis, Budapest, Hungary). The eluent

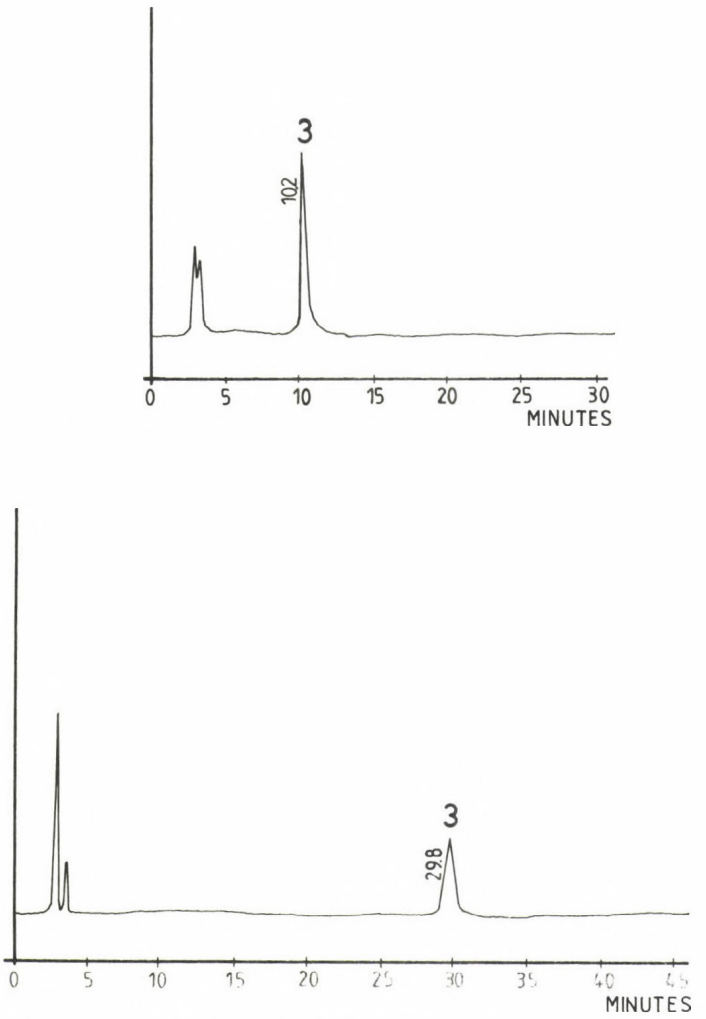


Fig. 3. The analysis of 20-hydroxyecdysone-22-acetate by HPLC, using C-18 as stationary phase.
Mobile phase: top chromatogram: solution No. 2.
bottom chromatogram: solution No. 1.
The number on the side of the peak indicates the retention time (in minutes). Detection at 254 nm

flow rate was 0.80 mL/min and isocratic elution was utilized. The elution of the ecdysteroids was monitored at 254 nm.

RESULTS

The purity of 20-hydroxyecdysone-22-acetate was controlled by HPLC as shown in Fig. 3.

Fig. 4 illustrates the analysis of the methanolic extract of Silene otites (L) Wib. using isocratic elution with solution No. 1.

Fig. 5 shows the separation of five ecdysteroids (1,20-dihydroxyecdysone; 20-hydroxyecdysone; 20-hydroxyecdysone-22-acetate; 2-deoxy-20-hydroxyecdysone; 2-deoxyecdysone). In this case, isocratic elution was performed using 1:1 methanol - triethylamine-phosphate buffer as the mobile phase. Fig. 6 represents the analysis of the methanolic extract of Silene otites (L) Wib. by HPLC using the same mobile phase as for Fig. 5.

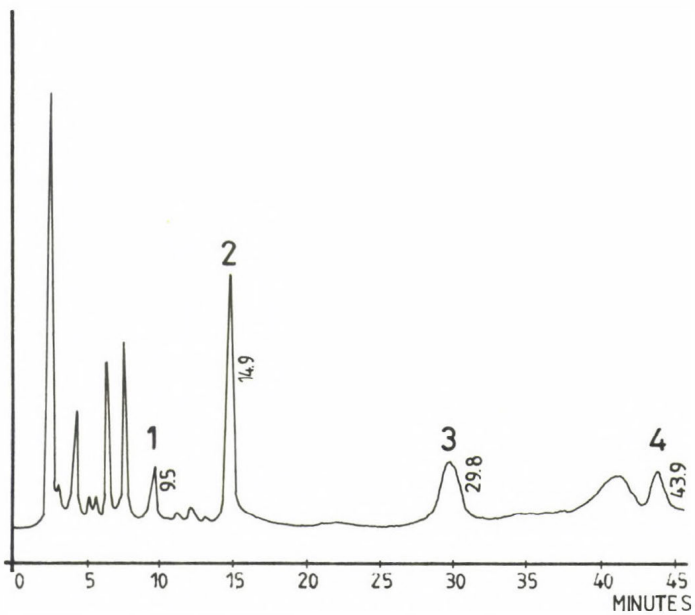


Fig. 4. Separation of the ecdysteroids in the extract of Silene otites (L) Wib. by HPLC, using C-18 as the stationary phase and solution No. 1. as the mobile phase. For peak identification see Fig. 2. The numbers on the side of the peaks indicate the retention times (in minutes). Detection at 254 nm

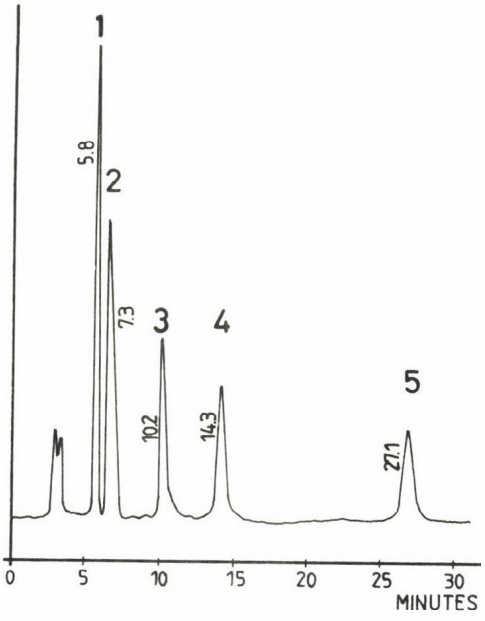


Fig. 5. Analysis of a standard mixture of five ecdysteroids, using C-18 as the stationary phase and solution No. 2 as the mobile phase. For peak identification see Fig. 2. The numbers on the side of the peaks indicate the retention times (in minutes). Detection at 254 nm

DISCUSSION

Several papers have been dealing with the analytical separation of ecdysteroids using various chromatographic methods. For routine screening of plant extracts and for the quality control of the products, thin-layer chromatography is still the method of choice. At the same time, high-performance liquid chromatography has also been introduced for the analyis of ecdysteroids. The ecdysteroids found in the insects were analyzed by HPLC /14, 15/ using C-18 stationary phase. In this case, the low ecdysteroid content of the samples made the utilization of a preliminary purification step advisable. The prepurification procedure could be omitted in our case, when the high concentration of ecdysteroids in the sample permitted their direct HPLC analysis. However, the complexity of ecdysteroid spectrum of

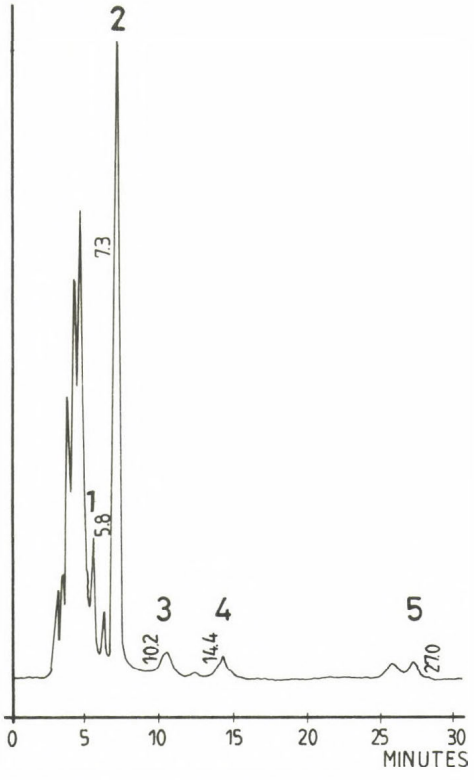


Fig. 6. Separation of the ecdysteroids in the extract of Silene otites (L) Wib. Conditions as in Fig. 5

Silene otites (L) Wib. strongly suggested both the investigation whether the peaks are homogenous, and the application of high organic modifier concentration in order to include late eluting peaks in the chromatograms. The separation of the peaks with lo k' values is possible when the ratio of the organic modifier vs. buffer concentration is relatively low, i.e. when the methanol concentration is kept at a possibly low level.

Experiments with the extract of the plant (Fig. 4) clearly demonstrated that 1,20-dihydroxyecdysone; 20-hydroxyecdysone; 20-hydroxyecdysone-22-acetate; 2-deoxy-20-hydroxyecdysone are present in Silene otites (L) Wib. At the same time, 2-deoxyecdysone can only be eluted with solution No. 2 used as the mobile phase.

For this reason, eluent No. 2 can be better used for the screening of extracts of Silene species for ecdysteroid components, and the analysis with eluent No. 1 is advisable only for two reasons:

1. the separation of highly polar components such as 1,20-dihydroxyecdysone from other polar impurities in the extract is better using eluent No. 1;

2. the separation of the early eluting peaks (such as e.g. 20-hydroxyecdysone) from the potentially existing other ecdysteroids is improved.

In the case of plant extracts where the ecdysteroid spectrum has not yet been studied, either both (No. 1 and No. 2) eluents should be applied, or gradient elution should be utilized. An additional possibility is given by ElRassi and Horváth /16/ who suggested the use of dipolar heterons in the eluent: a dipolar heteron can result in both the adequate differentiation of the early eluting components and the elution of the slow moving sample constituents in the same run.

In the case of quality control of the purified ecdysteroid preparations, isocratic elution is preferred with one of the adequate solvent systems. In the case of 20-hydroxyecdysone-22-acetate, eluent No. 1 seems to be rather sufficient, as demonstrated in Fig. 3.

The qualitative run presented here indicates that the isolation of 20-hydroxyecdysone-22-acetate from Silene otites (L) Wib. by the help of column liquid chromatography, DCCC and fractionated crystallization /13/ resulted in a highly pure substance.

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Chromatography '84
H. Kalász and L.S. Ettre (Eds)

SEPARATION OF STEROID ALLYLIC ALCOHOLS AND KETOALCOHOLS BY HPLC

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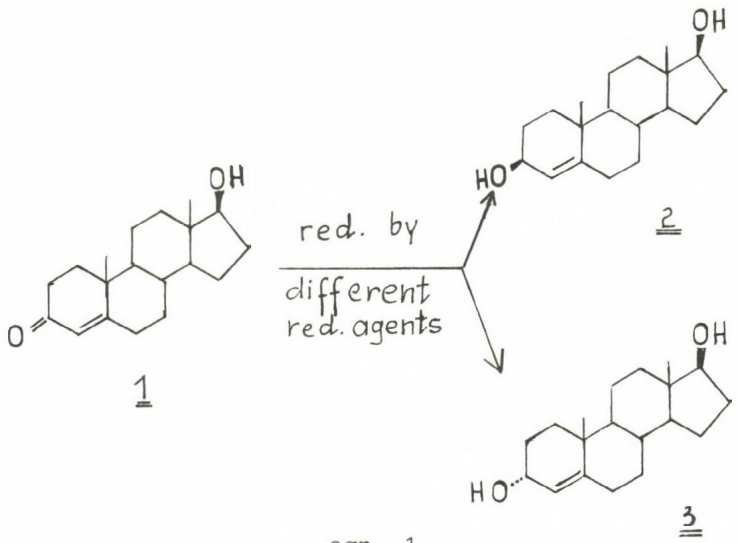
We earlier reported[1] the asymmetric hydrogenation of steroid ketones via hydrosilylation catalyzed by enantiomeric chiral rhodium-DIOP complexes and other reducing agents[2]. We later described[3] the separation of steroid ketoalcohols and diols by high-performance liquid chromatography.

The present paper describes easy routes for the separation of reduction products of steroid 4-en-3-ones (1, 4, 6). The reduction of testosterone (1) (eqn. 1) 2α-fluoroandrost-4-ene-3,17-dione (4) (eqn. 2) and 2α-fluoro-testosterone (6) (eqn. 3) by potassium tri-(R, S-sec-butyl) borohydride, and by rhodium 0-isopropylidene-2,3-dihydroxy-1,4-bis (diphenylphosphine)-butane+silane reducing agents was previously reported[4,5] to yield 3β- and 3α-allylic alcohols (2, 3, 5, 7, 8).

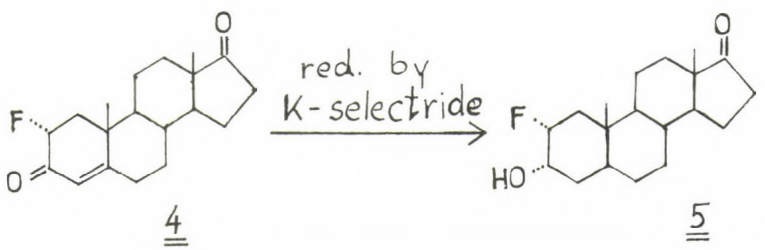
The products were separated by HPLC, using a Waters 244 liquid chromatograph, with an R401 differential refractometer and a Model 450 variable wavelength detector with a Whatman Partisil silica 10/25 column. The 3β- and 3α-allylic alcohols (2, 3) (eqn. 1) in the testosterone series were separated with the ether-hexane (3:1) solvent system.

As Fig. 1 shows, the retention time of 3β-hydroxytestosterone (2) was lower than those of the starting material (1) and the 3α-hydroxy isomer (3)

The chemo- and stereoselective reduction of 2α-fluoroandrost-4-ene-3,17-dione (4) (eqn. 2) by potassium tri-(R,S-sec-butyl)borohydride[4] produced only 2α-fluoro-3α-hydroxyandrost-4-en-17-one (5) which was separated from the starting material (4) with the hexane-propanol-2 (7:3) solvent system. The start-



eqn. 1



eqn. 2

ing material (4) was obtained with lower retention time (Fig. 2).

The 3β- and 3α-hydroxy isomers (7, 8) of 2α-fluorotestosterone (6) - obtained by different reductions of 2α-fluorotestosterone (6) (eqn. 3) - were successfully separated with the hexane-propanol (7:3) solvent system (Fig. 3). The results are similar to those seen with testosterone. The retention time of the 3β-hydroxy isomer (7) was lower than that of the 3α-hydroxy isomer (8). The retention time of the starting material (6) was between those of the 3α- and 3β-hydroxy isomers.

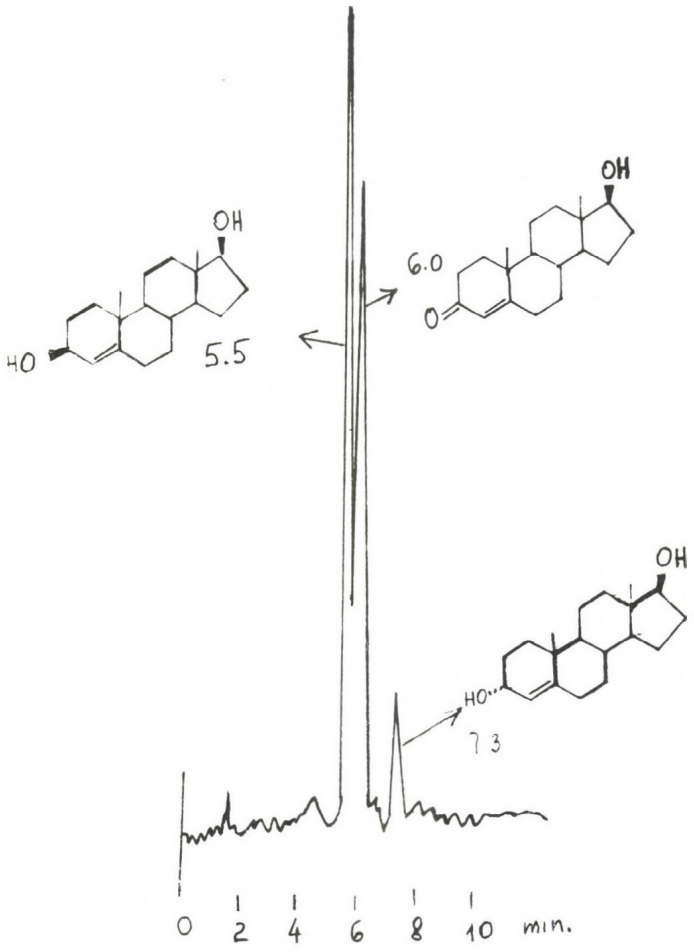


Fig. 1. Separation of testosterone derivatives

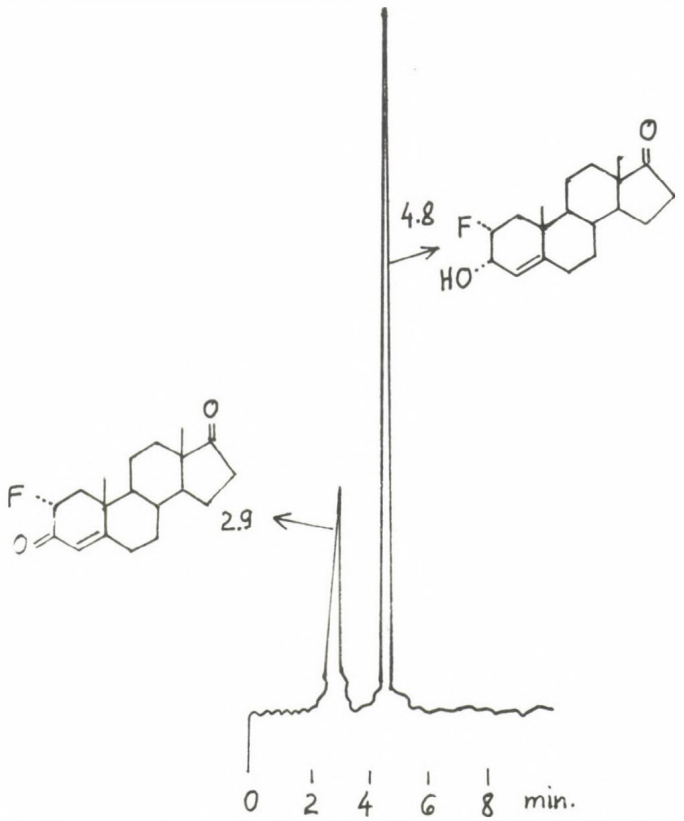
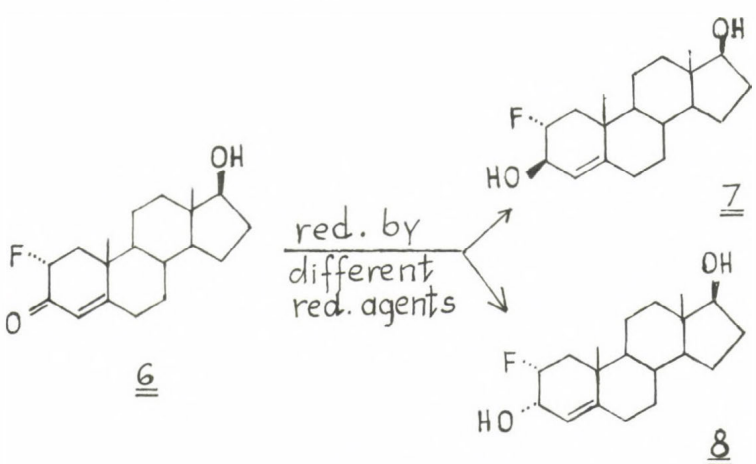


Fig. 2. Separation of 2α-fluoroandrost-4-ene-3,17-dione and 3α-hydroxy derivative



eqn. 3

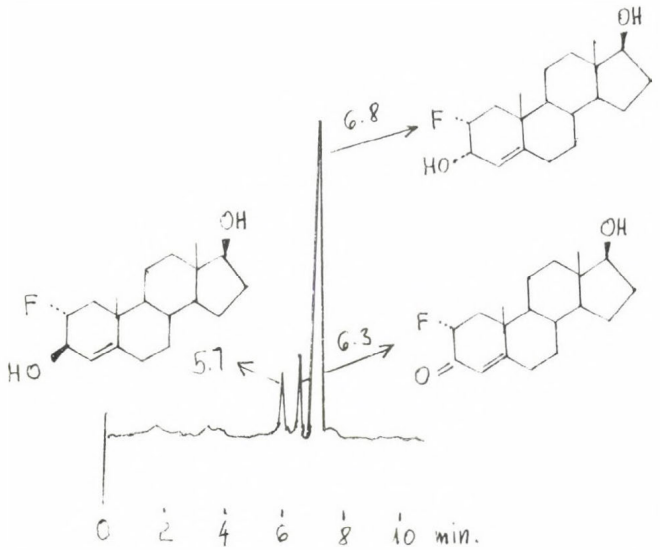


Fig. 3. Separation of 2α-fluorotestosterone and 3-hydroxy derivatives

CONCLUSION

In the separation of the 3β- and 3α-hydroxy isomers of both testosterone and 2α-fluorotestosterone the 3α-hydroxy isomers were the more polar, and they displayed higher retention times than the 3β-hydroxy isomers.

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HPLC OF SOME 8-AZA-12-KETOSTEROIDS AND THEIR OXIMES

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SUMMARY

HPLC separation of some biologically active diastereomeric 8-aza-12-ketosteroids (8-azagonane-12-on derivatives) and their oximes (all of them racemates containing three asymmetric carbon atoms in 9, 13 and 14 positions) is described.

INTRODUCTION

8-aza-12-ketosteroids (8-azagonane-12-on derivatives) and related compounds were synthesized first by Szántay and co-workers in 1977 /1/.

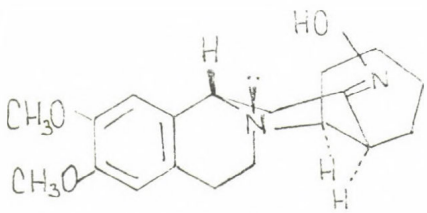
Owing to their significant physiological effects extended research was carried out in recent years for synthesizing new derivatives and for structure elucidations /2-6/.

Due to the bridgehead nitrogen atom in position 8 of the steroidlike ring system, 8-azasteroids can also be considered as condensed quinolizidines.

When 8-aza-12-ketosteroids are synthesized as above, diastereomer mixtures are usually formed whose composition depends on the reaction conditions used. In order to detect and determine the various diastereomers and parent ketones in the presence of each other HPLC seemed for us as the method of choice.

The HPLC procedures described below have been successfully applied to optimize large-scale manufacture, process control and specifications for some selected compounds of these series /7/.

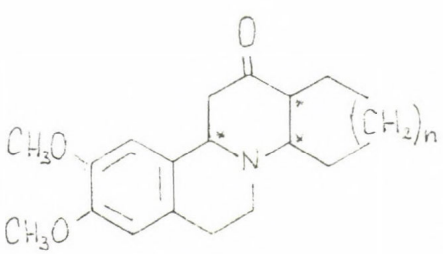
Application of these procedures is planned in clinical trials (determination of blood level, bioavailability, etc.) for a new, potent neuroleptic 8-azasteroid („EGYT-1623") which is under development.



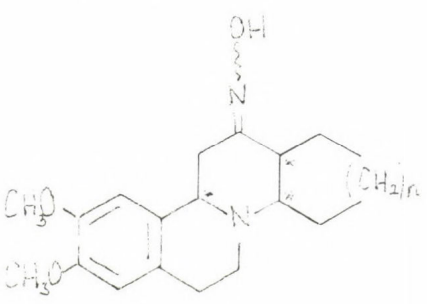
EGYT - 1623

CHEMISTRY OF 8-AZA-12-KETOSTEROIDS AND THEIR OXIMES

The studied 8-aza-12-ketosteroids (I) and their oximes (II) contain three asymmetric carbon atoms (C_9, C_{13} and C_{14}). Eight diastereomeric ketones and sixteen oximes (included E- and Z-isomers) are therefore possible (four and eight racemates, respectively) in each series (n = 1, 2, etc.).



I

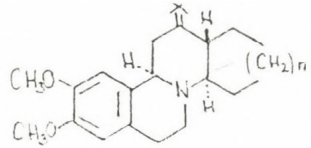
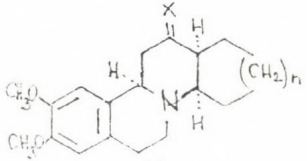
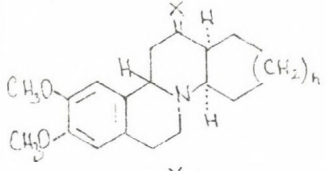
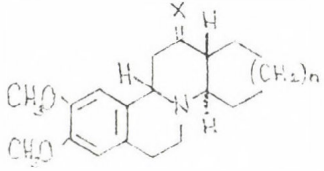


II

According to B/C and C/D ring anellations four different ring systems (configurations) are possible (see Table 1).

In the individual series (n = 1, 2, etc.) all configurations cannot synthetically obtained, due to the thermodynamic instability of certain ring systems of given D-ring size.

Table 1. Ring systems and configurations of the investigated 8-azasteroids

Configuration	Stereostructure*	Ring anellations B/C	Ring anellations C/D	D-ring	Abbreviations ketones	Abbreviations oximes
Normal		trans	trans	six-membered (n=2)	6a	6a-OX(E)
Allo		trans	cis	five-membered (n=1)	5a	5a-OX(E) 5a-OX(Z) **
Epiallo		cis	cis	five-membered (n=1)	5b	5b-OX(E) 5b-OX(Z) **
				six-membered (n=2)	6b	6b-OX(E) 6b-OX(Z)
Pseudo		cis	trans	six-membered	6c	6c-OX(E)

* Stereostructures shown in Table 1 describe only H-14 enantiomers of the investigated racemates.

** E/Z assignment for 5a-OX and 5b-OX is not yet unambiguously proven.

In the case of oximes both E and Z diastereomers can be formed in the oximation reaction of the parent ketone. The ratio of E/Z diastereomers formed as above depends both on the parent ketone and the reaction conditions.

Ring systems and configurations of the investigated compounds are summarized in Table 1.

EXPERIMENTAL

HPLC apparatus

A Pye-Unicam LC - XP Liquid Chromatographic System (containing an Altex 101 A pump, a Rheodyne 7125 precision loop injector with a 10 µl loop, and a PU - 4020 variable-wavelength UV - detector with 8 µl flow cell) and a Hewlett-Packard 3390 A Reporting Integrator were used.

Reagents

Water was double distilled and stored in glass. Hexane, acetonitrile, methanol, ethanol, chloroform and isooctane were HPLC grade (Merck „LiChrosolve" quality). Diethylamine and conc. ammonia (REANAL) were reagent grade.

The „steroid-base" chemical name of the compounds listed in Table 1. are as follows.

5a: rac.-2,3-Dimethoxy-8-aza-13α-gona-1,3,5(10)-trien-12-one

5b: rac.-2,3-Dimethoxy-8-aza-9β,13α-gona-1,3,5(10)-trien-12-one

6a: rac.-2,3-Dimethoxy-8-aza-D-homo-gona-1,3,5(10)-trien-12-one

6b: rac.-2,3-Dimethoxy-8-aza-D-homo-9β,13α-gona-1,3,5(10)-trien-12-one

6c: rac.-2,3-Dimethoxy-8-aza-D-homo-9β-gona-1,3,5(10)-trien-12-one

5a - OX (E and Z): rac.-(ξ)-2,3-Dimethoxy-8-aza-13α-gona-1,3,5(10)-trien-12-one-oxime

5b - OX (E and Z): rac.-(ξ)-2,3-Dimethoxy-8-aza-9β,13α-gona-1,3,5(10)-trien-12-one-oxime

6a - OX (E): rac.-(E)-2,3-Dimethoxy-8-aza-D-homo-gona-1,3,5(10)-trien-12-one-oxime

6b - OX (E): rac.-(E)-2,3-Dimethoxy-8-aza-D-homo-9β, 13α-gona-1,3,5(10)-trien-12-one-oxime („EGYT - 1623")

6b - OX (Z): rac.-(Z)-2,3-Dimethoxy-8-aza-D-homo-9β,13α-gona-1,3,5(10)-trien-12-one-oxime

6c - OX (E): rac.-(E)-2,3-Dimethoxy-8-aza-D-homo-9β-gona-1,3,5(10)-trien-12-one-oxime

Chromatographic conditions

Columns, mobile phases and flow rates used are given in Tables 2 and 3. Isocratic elution technique was used. The columns were thermostated at 30°C.

Procedure

Synthetically prepared high-purity grade bulk materials (obtained according to [1], [2] and [3]) were dissolved in the same solvent system as used for the mobile phase. The concentration range of the samples was 0.2 - 0.3 mg/ml and 10 µl aliquots were injected. The mobile phases were filtered through G_4 sintered glass filter and ultrasonicated before use.

RESULTS AND DISCUSSION

1. 8-aza-12-ketosteroids

Chromatographic conditions are summarized in Table 2.

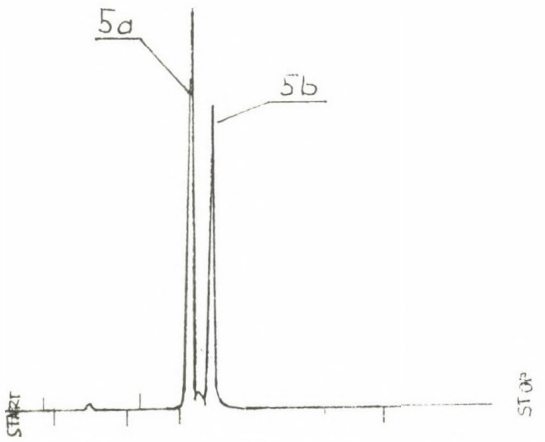


Fig. 1. Reversed phase HPLC of compounds 5a and 5b

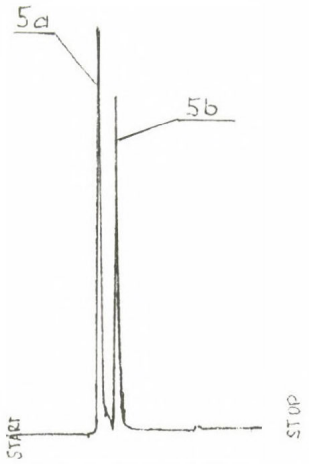


Fig. 2. HPLC of compounds 5a and 5b in a normal-phase system

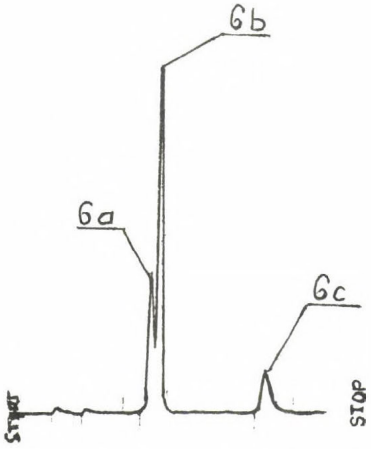


Fig. 3. Reversed phase HPLC of compounds 6a, 6b and 6c

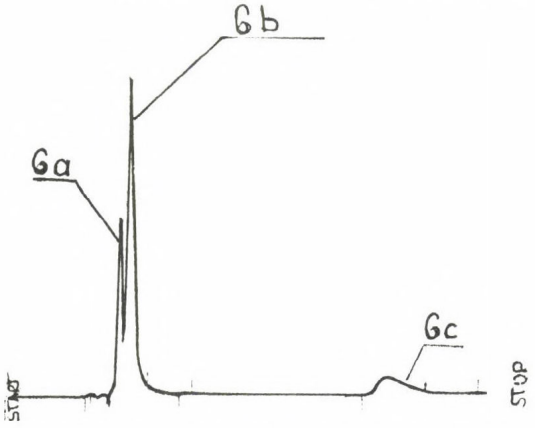


Fig. 4. HPLC of compounds 6a, 6b and 6c in a normal-phase system

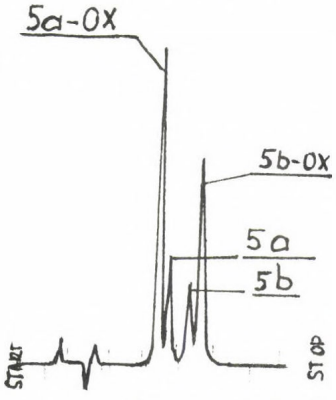


Fig. 5. Reversed phase HPLC of compounds 5a, 5b, 5a-OX (E or Z) and 5b-OX (E or Z)

Table 2. Chromatographic conditions for 8-aza-12-ketosteroids

Compounds to be separated	Chromatographic conditions				N° of Figure
	Column	Mobile phase (v/v)		Flow rate (ml/min)	
5a, 5b	Du Pont Zorbax TMS 250x4.6 mm ID	Acetonitrile Water Ammonia (1% soln)	60 40 0.2	0.61	1
5a, 5b	Beckman Ultrasphere Si 5 µm 250x4.6 mm ID	Isooctane Chloroform Ethanol Diethylamine	40 55 5 10^{-5}	1.01	2
6a, 6b, 6c	Du Pont Zorbax TMS 250x4.6 mm ID	Acetonitrile Water Ammonia (1% soln)	47.5 52.5 0.3	1.01	3
6a, 6b, 6c	Beckman Ultrasphere Si 5 µm 250x4.6 mm ID	Hexane Chloroform Methanol Diethylamine	56 40 4 10^{-5}	1.01	4

2. 8-aza-12-ketosteroid oximes

In the case of oximes the separation of diastereomers and detection of the parent ketones present as impurities were needed.

The detection of the 5a and 5b parent ketones in the corresponding oximes can be achieved in both reversed-pase and normal-phase systems (Figs 5 and 6). Higher resolution and better elution order was obtained, however, in the latter case.

As Fig. 7. shows, good separation can be obtained for the diastereomeric oximes with 6-membered D-ring and even better for 6b - OX(E) and 6b - OX(Z).

In this case, however, the 6a and 6b ketones cannot be separated.

Table 3. HPLC of 8-aza-12-ketosteroid oximes and their parent ketones

Compounds to be separated	Chromatographic conditions			N° of Figure
	Column	Mobile phase (v/v)	Flow rate (ml/min)	
5a		Acetonitrile 60		
5b	Du Pont	water 40		
5a-OX(E)	Zorbax TMS	Ammonia 0.2		
5a-OX(Z)		(1% soln)	1.01	5
5b-OX(E)	250x4.6 mm ID			
5b-OX(Z)				
5a				
5b	Beckman	Isooctane 40		
5a-OX(E)	Ultrasphere Si	Ethanol 5		
5a-OX(Z)	5 μm	Chloroform 55	1.41	6
5b-OX(E)	250x4.6 mm ID	Diethylamine 5×10^{-5}		
5b-OX(Z)				
6a-OX(E)	Beckman	Chloroform 90		
6b-OX(E)	Ultrasphere Si	Methanol 10		
6b-OX(Z)	5 μm	Diethylamine 10^{-3}	1.01	7
6c-OX(E)	250x4.6 mm ID			
6a				
6b				
6c	Du Pont	Acetonitrile 54.5		
6a-OX(E)	Zorbax TMS	Water 45.5	1.01	8
6b-OX(E)		Ammonia 0.08		
6b-OX(Z)	250x4.6 mm ID	(1% soln)		
6c-OX(E)				

If the detection of 6a and 6b ketones is needed in the diastereomeric oximes of 6-membered D-ring, the reversed phase HPLC system is to be applied (Fig. 8).

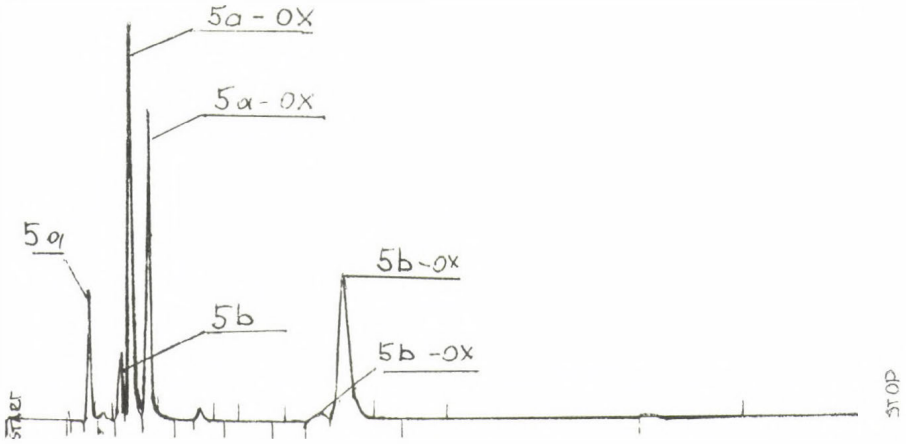


Fig 6. HPLC of compounds 5a, 5b, 5a-OX (E and Z) and 5b-OX (E and Z) in a normal-phase system

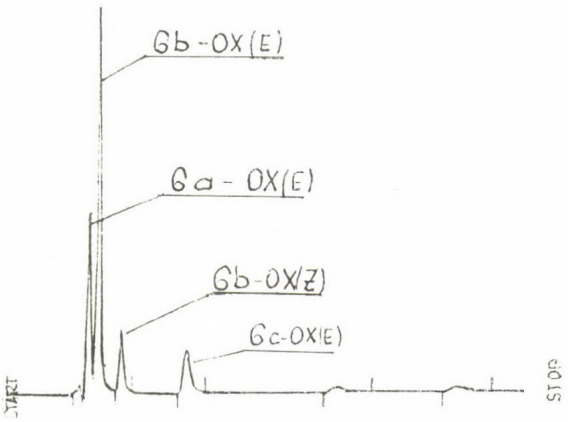


Fig. 7. HPLC of compounds 6a-OX(E), 6b-OX(E), 6b-OX(Z) and 6c-OX(E)

Because the UV-absorptivity of the diastereomers having identical composition was found very comparable, the diastereomeric purity can be easily computed from the corresponding peak areas.

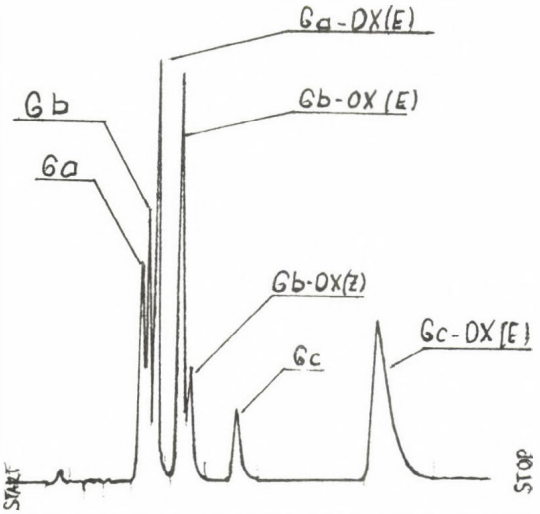


Fig. 8. Reversed phase HPLC of compounds 6a, 6b, 6c 6a-OX(E), 6b-OX(E), 6b-OX(Z) and 6c-OX(E)

As it has been mentioned above all the studied compounds were racemates. Investigations are in progress to separate them into their enantiomers.

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METHOD FOR THE DETERMINATION OF NATURALLY OCCURRING MACROCYCLIC TRICHOTHECENE TOXINS

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ABSTRACT

An improved three step (TLC, GLC, HPLC) analytical procedure for the determination of macrocyclic trichothecenes in various cereals is described. TLC with Kieselgel plate and an isopropanol-chloroform solvent system, capillary GLC and HPLC performed with reversed-phase column was used. Compounds were detected with an ultraviolet detector (254 nm). Recovery of added toxins were determined (Satratoxin H 78%, Satratoxin G 72%, Verrucarin J 68%) at 0.1 mg/kg level. Five field samples were examined and the correct macrocyclic trichothecene content was determined. The described method is simple, reliable and suitable for the determination of naturally occurring macrocyclic trichothecenes below the health hazard concentration.

INTRODUCTION

Macrocyclic trichothecene mycotoxins are toxic metabolites produced by fungi, including Stachybotrys and Myrothecium /1, 2/ (Fig. 1.). These fungi are known as plant-pathogenic fungi /3/.

Various reports have appeared dealing with macrocyclic trichothecene related mycotoxicosis (stachybotryotoxicosis) /4 - 7/. The occurrence of such type of disease has been described in various part of the world such as e.g., in South Africa /7/, Finland /8/, the Soviet Union /9/, France /10/, and Hungary / 5, 6/. Strains originated from India and Egypt also

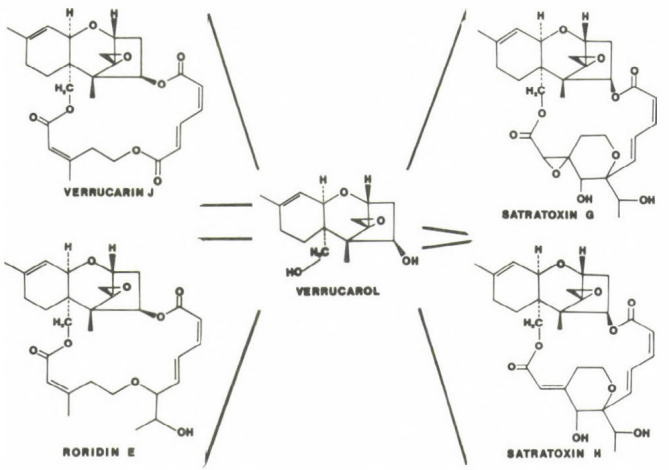

Fig. 1. The chemical structure of naturally occurring macrocyclic trichothecenes

produced stachybotrys toxins /11/. The only available method for the determination of these toxins in cereal samples was published by Stack and Eppley /12/. However, their method is not enough selective and sensitive.

A new analytical method is described in this paper employing thin-layer chromatography (TLC), gas chromatography (GC) and high-performance liquid chromatography (HPLC). For the confirmation of the analysis results brine shrimp bioassay was used. Off-line combination of HPLC with other techniques (TLC, GC or bioassay) appears to be particularly advantageous for an unambiquous determination of macrocyclic trichothecenes.

EXPERIMENTAL

Standards

Satratoxin G and H were prepared in our laboratory and were checked by MS and NMR. Verrucarin J was a gift from Prof. Tamm of the University of Basel. Roridin E was a gift from Prof. Jarvis of the University of Maryland (USA).

Sample Extraction and Clean-up Produce

The sample was grinded into small pieces and 50 g were put into an Erlenmeyer flask and extracted twice with 200 ml methanol for four hours. The two extracts were combined and evaporated to 50 ml. 20 ml water was added to the extract and it was washed with 3 x 30 ml of petroleum ether. The toxin-containing methanolic phase was evaporated on a water bath. The part containing the residual water was extracted with 3 x 10 ml of ethyl acetate and the ethyl acetate phase was evaporated to dryness. The residue was dissolved in 1 ml of methanol and 1 ml of water was added (methanol-water 1+1). This mixture was passed through a Sep-Pack C_{18} cartridge (Waters Associates Inc., Milford, Mass. USA) which was then washed with 2 ml of 1:1 methanol-water. The examined toxins were eluted with 3 ml of 8:2 methanol-water from the cartridge which was regenerated with 5 ml of ethanol and equilibrated with 5 ml of 1:1 ethanol-water before use. The toxin-containing eluent was evaporated to dryness in a nitrogen stream at 80 ^{o}C. The residue was redissolved in 100 µl of methanol and 20 µl aliquot was injected.

TLC

Kieselgel, 60 10 x 10 cm HPTLC (Merck, Darmstadt, FRG), plates were used for TLC analysis. The solvent system was 94:6 (v/v) chloroform-isopropanol. Compounds can be detected by the use of UV light (254 nm) or the plates can be sprayed with 1% 4-(p-nitrobenzyl)-pyridine solution, dried at 150 ^{o}C for 30 minutes, and sprayed with 10% tetraethylene pentamine reagent. Toxins give bluish-violet spots on a white background.

GLC

Fifty microliters of a 0.5 N sodium methoxide reagent were added to the dry residue for transmethylation (Fig. 2.):

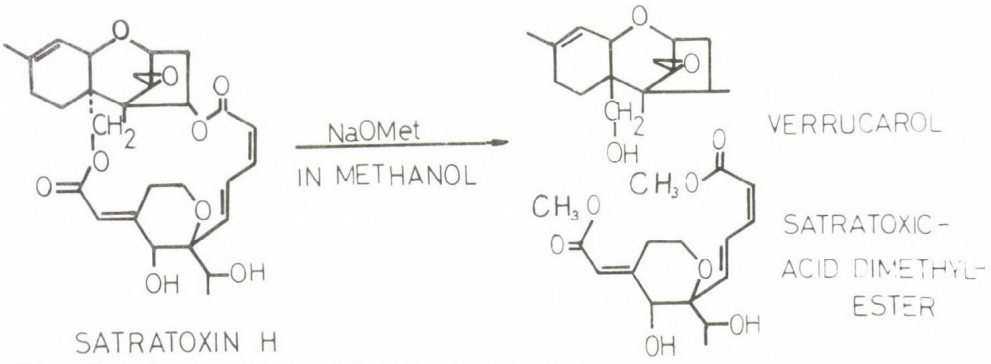

Fig. 2. The reaction of transmethylation

The mixture was placed for 15 minutes into a block thermostat at 60 °C. At the end of the reaction 60 µl of methanol containing 0.5 N HCl were added to the mixture to neutralize the base and the mixture was evaporated to dryness. Then 100 µl of bis (trimethylsilyl) trifluoroacetamide (BSTFA) (Sigma, St. Louis, Mo, USA) reagent was added and it was placed again into a block thermostat at 60 °C for 15 minutes. For the investigation of the bis-trimethylsilyl ether derivate of verrucarol, a glass capillary coated with SE-52 stationary phase was used (10 m x 0.30 mm i.d.). Column temperature was programmed from 160 to 220°C at 4 °C/min. Split ratio was 1:10.

HPLC

The high-performance liquid chromatographic (HPLC) separations were performed with a Waters instrument on a 5 µm C_{18} Poligosyl 60 D column (25 cm x 4.6 mm) (Macherey-Nagel, Duren, FRG). Gradient elution was used: eluent A was 20:80 (v/v) methanol-water while eluent B was 80:20(v/v) methanol-water. The running time was 20 min. The detection of the macrocyclic trichothecene toxins was carried out by a Waters Model 440 ultraviolet (UV) detector set at 254 nm.

RESULTS

The R_f values of satratoxin G and H and verrucarol were 0.53, 0.43 and 0.30 respectively. Figure 3 shows the gas chromatogram of a sample containing the trichothecene skeleton (verrucarol).

Figure 4 shows the HPLC chromatogram of some samples without transmethylation.

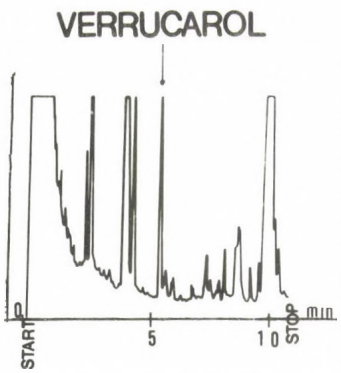

Fig. 3. GLC chromatogram of straw extract after transmethylation

The sensitivity of the TLC method was 0.3 mg/kg for the determination of the verrucarol content of the sample. It was 0.2 mg/kg was for satratoxin G and H respectively.

The sensivity of the GLC method was 0.05 mg/kg for satratoxins G and H and 0.015 mg/kg for roridin E.

Some results of the investigation of field samples are presented in Table 1.

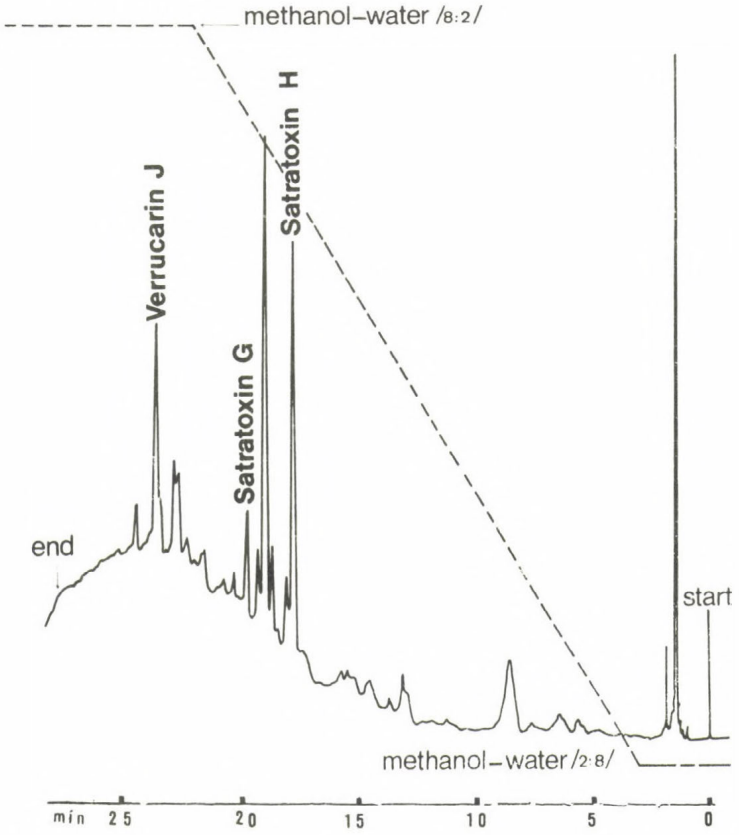

Fig. 4. HPLC chromatogram of straw extract

Table 1. Natural occurrence of macrocyclic trichothecenes in field samples

Sample	Source	Satratoxin G	Satratoxin H	Verrucarin J
		mg/kg		
Budapest 1	straw	0.020	0.75	0.035
Budapest 1	straw	0.010	0.050	0.025
Vaja	straw	0.060	0.140	0.085
Kecskemét	straw	0.020	0.065	0.030
Puszta	hay	0.015	0.045	0.020

Quantitation and Recovery

For the quantitation, calibration with external standard was performed. In order to get the linear relationship between the toxin amount and the peak area, different amounts of macrocyclic trichothecene toxins were injected (e.g., 10, 20, 50, 75 and 100 ng).

Recovery experiments were carried out by adding 5 µg satratoxin G and H and verrucarin J to the samples. Nine parallel experiments were carried out. The recovery was 78% for satratoxin G and 68% for verrucarin J.

DISCUSSION

Earlier most sensitive methods /12/ used for the determination of trichothecene macrocyclic toxins permitted the determination of these toxins over the 0.20 mg/kg concentration level. Considering that in many cases a concentration level of 0.05 - 0.10 mg/kg may cause serious disease in animal husbandry a more sensitive analytical method is needed. Using the method of Stack and Eppley /12/ it is only possible at the end of the total analytical procedure to obtain information about the toxicity of the sample. An advantage of the method proposed in the present paper is the fact that after TLC or GLC it is possible to classify the sample in three categories: non toxic, slightly toxic, or strongly toxic. An absence of verrucarol indicates the non-toxicity and HPLC investigation is not needed. Naturally, for the determination of verrucarol GLC is preferred against TLC.

For the control of the HPLC results a second transmethylation may be used. In this case the presence of trichothecene skeleton may be confirmed using standards.

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DETERMINATION OF A NEW ANTIATHEROSCLEROTIC DRUG (CH-123) IN HUMAN SERUM USING MICRO HPLC

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SUMMARY

A sensitive micro high-performance liquid chromatographic precolumn method is described for the determination of a new antiatherosclerotic drug (CH-123) and its major metabolite MZ-672) in human serum. The serum was injected directly without any sample pretreatment. Internal standard was used to eliminate sample size fluctuation. The method was applied to single-dose studies with three volunteers.

INTRODUCTION

In the past several years there has been an increasing interest in the use of microbore columns (1-7). Various advantages of miniaturized HPLC have been mentioned, e.g., the reduction of the cost of packing material and solvents used as the mobile phase; low flow rates should facilitate direct interfacing to other special detectors /e.g. mass-spectrometer (8-10), flame photometer (11), Fourier transform IR (12)/; they can reduce the detection limit (13) and increase the speed of separation (2, 14).

As peak broadening in the column is small, micro-LC is especially suitable for trace analysis (13, 16-18).

A micro high-performance liquid chromatographic method described by us earlier (15) was applied to single-dose studies

of a new antiatherosclerotic drug (CH-123) and its metabolite (MZ-672) levels in human serum using UV detection.

As the injection volume is much smaller in micro-HPLC than in ordinary HPLC, we have adopted a micro precolumn sample enrichment (16) for direct injection of body fluids instead of the conventional solvent extraction. There was used S-353 as internal standard to eliminate sample size fluctuation.

MATERIALS AND METHODS

Instrument and columns

The Familic-lOON micro HPLC (JASCO, Japan) was used in our work. The pump is of syringe type and the flow rate can be selected from 1 to 29 µl/min. For the detection a conventional UV detector, the model UVIDEC-II (JASCO) can be used. The 0.3 µl quartz capillary cell was fixed on a micro cell casette. For sample injection a 0.3 µl loop injector was used.

The micro separation column was packed by the slurry technique as described earlier (1) with Zorbax C8 (DuPont). The column was a teflon tube, 14 cm x 0.5 mm i.d.

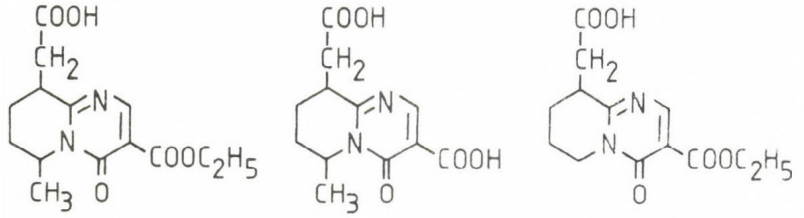
The micro precolumn for enrichment was made by packing LiChrosorb C8 (10 µm, Merck) in a teflon tube, 2 cm x 0.5 mm i.d. The micro precolumn was connected to the separation column with 10 mm x 0.35 mm i.d. stainless-steel tubing.

Reagents

Analytical reagent grade chemicals were used without further purification. All solutions were prepared by using distilled and deionized water.

For the standard samples CH-123, MZ-672 and the internal standard S-353 (from CHINOIN) were dissolved in a 0.02M acetate buffer (pH=3). The stock solutions (200 µg/ml) were stored at $4^{o}C$. Serial dilution in the buffer for serum standard curves of CH-123 and MZ-672 were made daily so that 100 µl of the solution would correspond to a concentration range of 0.125-2.5

μg/ml serum. The final concentration of S-353 used as the internal standard was 1.25 g/ml (50 ng/injection).



CH-123 MZ-672 S-353

The serum was adjusted with 2M HCl solution to pH=3. The mobile phase used consisted of a 0.05M acetate buffer (pH=3) containing 20% methanol as the organic modifier.

Sample injection into the precolumn

The micro precolumn was washed with 25 μl buffer before the sample injection. 50 μl of a human serum adjusted to pH=3 was injected into the column for about 5 min with a syringe. Consequently the column was washed with 25 μl buffer and connected to the separation column.

At lower concentrations 100 μl serum was injected.

In vivo studies

Three healthy volunteers (one male and two females) were fasted overnight and received a sigle oral dose of CH-123 (300 mg capsule).

Blood samples were collected by venous puncture in vials at 0, 0.5, 1, 2, 4 and 6 hr following the dose. They were centrifuged within 10 min at 1000 g (4^{o}C) to obtain the serum which was then stored at -40^{o}C until the analysis.

At the analysis the internal standard S-353 (50 ng/inj.) was added to the serum. Then the sample was adjusted to pH=3 with HCl solution and was injected into the precolumn.

RESULT AND DISCUSSION

MZ-672, the internal standard S-353 and CH-123 gave symmetrical peaks under the described conditions with retention times of 12.5, 16.2 and 26 min, respectively.

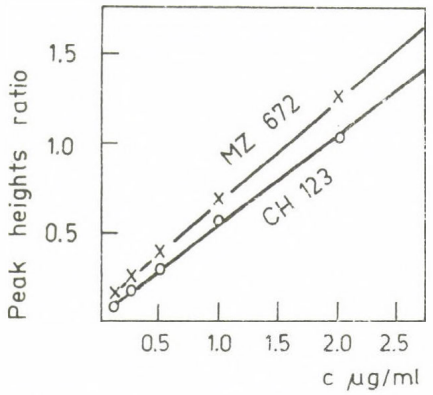


Fig. 1. Calibration curves of CH-123 and MZ-672; 50 ng/inj. S-353 was used as the internal standard (for conditions see Fig. 2.)

The calibration curves in serum were found to be linear in the concentration range of 0.125 - 2.5 µg/ml (Fig. 1.). The data are best described by a linear equation for CH-123: $Y_1 = 0.475X_1 + 0.046$ (r=0.9957),

where X_1 is the concentration of CH-123, in µg/ml, and Y_1 is the peak height ratio of CH-123 to the internal standard S-353. The same equation for MZ-672 is: $Y_2 = 0.598X_2 + 0.052$ (r=0.9963).

The coefficients of variation (C.V.) measured from replicate analyses (n=3) at 1 µg/ml was 6.1% for CH-123, 4.1% for MZ-672 and at lower concentration, 0.1 ug/ml, 14.7% for CH-123 and 16.8% for MZ-672.

The detection limit was improved up to 50 ng/ml by the injection of 100 µl serum.

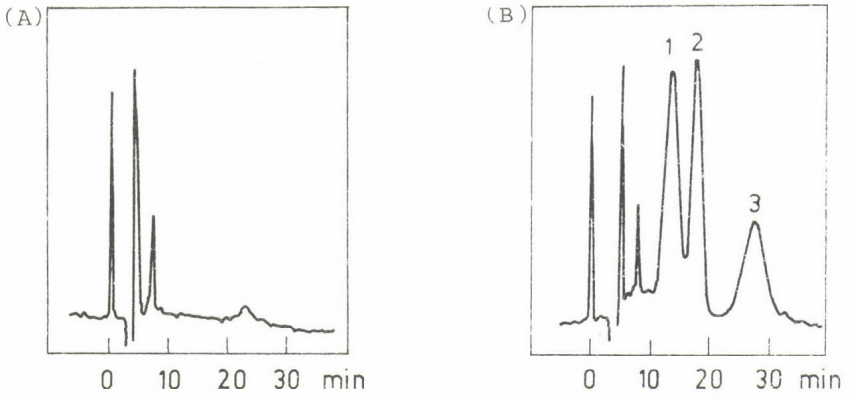


Fig. 2. (A) Chromatogram of 100 µl directly injected human serum, (B) Chromatogram of 50 µl of a human volunteer serum sample collected 1h after a single dose of CH-123. Internal standard S-353 (peak 2) = 50 ng/inj; calculated CH-123 (peak 3) concentration=0.69 µg/ml; calculated MZ-672 (1 peak) concentration: 1.77 µg/ml. Conditions: flow rate, 10 µl/min; detection, UV at 302 nm, 0.005 AU; mobile phase, 0.05M acetate buffer containing 20% methanol.

Fig. 2.A. shows the chromatogram of 100 µl human blank serum which was injected into the precolumn as described above. It is evident that no endogenous peaks interfere.

A chromatogram of patient serum 1h after administration (volunteer who had received 300 mg of CH-123) is shown in Fig. 2.B. The calculated concentrations are 0.69 µg CH-123/ml serum and 1.77 µg/ml MZ-672/ml serum.

The concentration versus time curves obtained after single dose CH-123 are shown in Fig. 3. The maximum levels in serum were found 0.71 ± 0.03 µg CH-123/ml and 2.20 ± 0.68 µg MZ-672/ml respectively at one hour after intake. At 6h after intake there was no CH-123 in the serum and the metabolite concentration was also low(about 0.06 µg/ml).

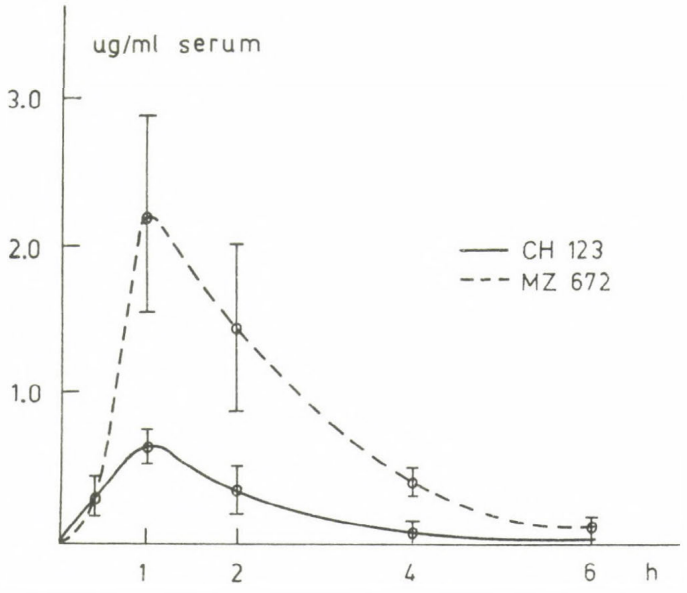


Fig. 3. Mean serum concentration of CH-123 and MZ-672 after a single oral dose of CH-123 (300 mg) (n=3)

CONCLUSION

A new simple micro-HPLC precolumn method for CH-123 has been developed. As demonstrated it is applicable to single-dose pharmacokinetic studies of this new antiatherosclerotic drug and its metabolite MZ-672 in serum.

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TUFTSIN: SYNTHESIS, PURIFICATION AND BIOLOGICAL PROPERTIES

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The tetrapeptide tuftsin with the structure as shown in Table 1 (compound I) features a broad spectrum of biological activity. It stimulates phagocytosis, is capable of an anti-tumoral effect, etc. /1, 2/. However, the data related to its biological capabilities are highly inconsistent, this being associated with the purity of the synthetic compounds used for biological tests /3/.

Particularly complex is the separation of tuftsin from its diastereomeric analogues formed as a result of partial racemization in the process of chemical synthesis. We have shown (Table 1) that some diastereomeric analogues (such as Compound VI) may manifest an effect opposite to that of the natural peptide, and, as a consequence, their presence may affect the activity of the compound. The methods of ion-exchange and gel chromatographies generally used for purifying synthetic peptides fail to provide for the separation of diastereomeric impurities. The present report discusses the possibilities of using high-performance liquid chromatography and hydrophobic ion-exchange chromatography for the separation of diastereomeric tuftsin analogues, with particular reference to the latter method as being technologically less involved but promising.

We synthesized tuftsin and its diastereomeric analogues following the scheme as shown in the flow diagram (Fig. 1) Passed through a carboxymethylcellulose-based purification step, followed by gel filtration, and identified as an individual (homogeneous) product by thin-layer chromatography and high-voltage paper electrophoresis, Compound VII was shown

Table 1. Tuftsin and its diastereomeric analogues: some biological, physical, and chemical properties

Sl Nos.	Compound	Phagocytic index*	High-performance liquid chromatography** k′		Hydrophobic ion-exchange chromatography*** k′		$(\theta)\cdot 10^{-3}$ $\frac{deg\cdot cm^2}{mole}$ ****	Ref.
I	Tuftsin (HThr·Lys·Pro·ArgOH)	90	1.5	0	3.6	0	-60	/4/
II	(D Arg^4)-tuftsin	70	4.0	4.3			-35	/5/
III	(D Pro^3)-tuftsin	68	4.3	4.9	4.4	0.9	+10	/5/
IV	(D Lys^2)-tuftsin	67	3.9	4.1			-20	/5/
V	(D Thr^1)-tuftsin	61	3.2	2.9			-50	/5/
VI	(D Thr^1·D Lys^2)-tuftsin	25	2.9	1.9	4.9	1.0	-55	
VII	Tuftsin (prior to purification by HPLC)	74	-	-	-	-	-	
	Control	63	-	-	-	-	-	

* Quantity of latex microsphere-phagocytizing peritoneal macrophage cells from SVA strain mice, % of the total number of cells

** Zorbax C-8 column, 5x250 mm, d = 5 μm, pH = 2.45, 0.1 M ammonium phosphate buffer, $T \simeq 20^{\circ}C$

*** Soloza K 30/40 packed column, 4x300, d = 90-125 μm pH = 8.0, 0.15M borate buffer, $T \simeq 20^{\circ}C$ /6/

**** Ellipticity of aqueous peptide solution at λ = 195 nm.

Fig. 1. Flow diagram of the synthesis of tuftsin and its stereoisomer analogues

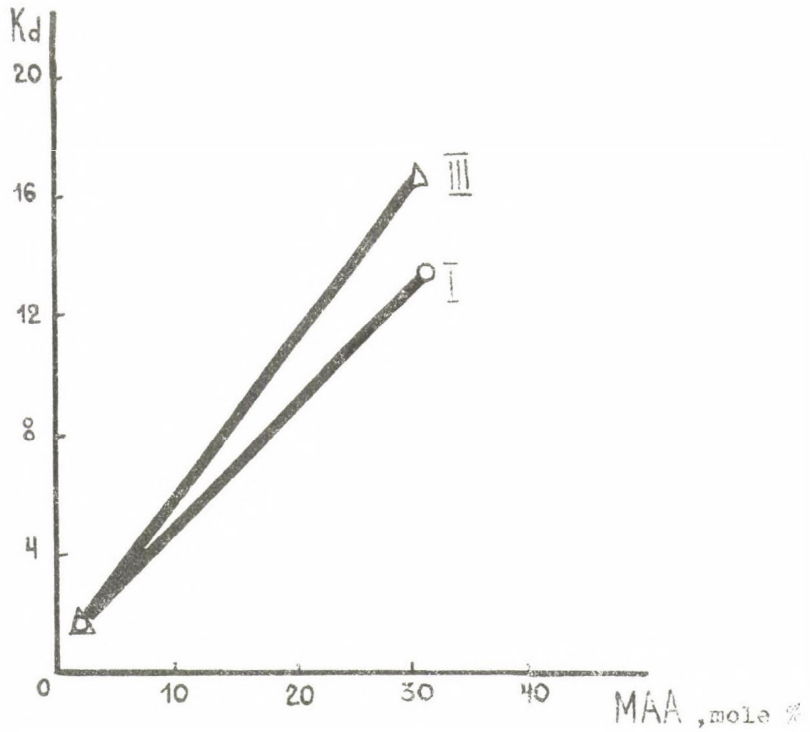

Fig. 2. Distribution coefficients (K_d) of tuftsin stereoisomers I and III versus methacrylic acid content (MAA, mole %) in Soloza K sorbents at constant butyl methacrylate content, pH = 8.0, 0.1M borate buffer

to contain impurities when analyzed by high-performance liquid chromatography. Some of the impurities may be identified as diastereomeric tuftsin analogues. The biological activity of Compound VII was found to be lower than that of the individual product obtained upon purification by high-performance liquid chromatography (Table 1, Compounds I and VII).

As may be seen from Table 1, diastereomeric tuftsin analogues are easily separable from tuftsin as such by the use of high-performance liquid chromatography, with a specific correlation observable between the capacity factors k' and the values of /θ/ at = 195.0 nm, obtainable from the CD spectra.

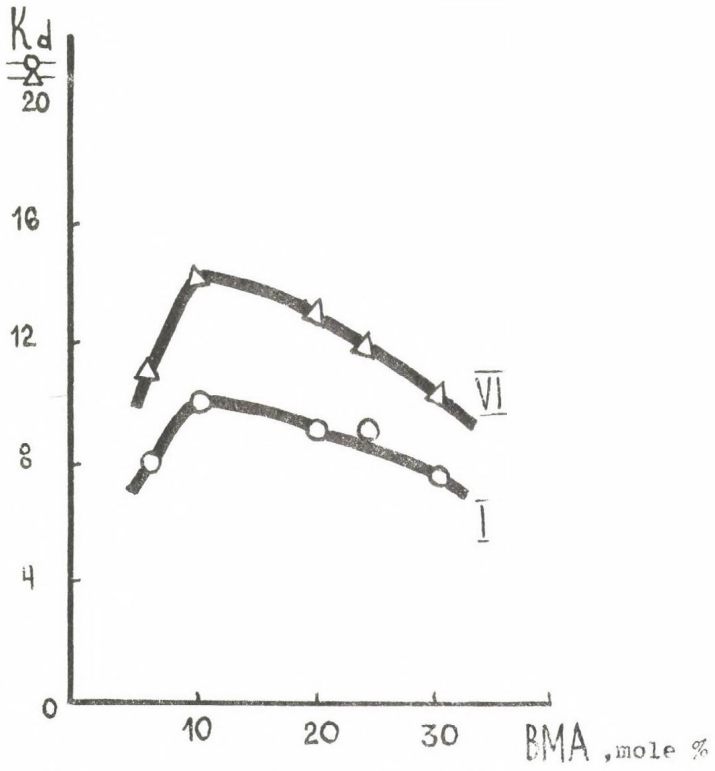

Fig. 3. Distribution coefficients of tuftsin stereoisomers I and VI versus Soloza K sorbent composition, pH = 8.0, 0.15M borate buffer, t = 20°C

Shown by CD data to have a conformation differing from that of tuftsin (positive Cotton effect). Compound III is the easiest to separate from Compound I Apparently, it is the difference in hydrophobicity of the most stable conformations of diastereomeric tuftsin analogues that enables their separation by high-performance liquid chromatography techniques.

We have also shown that diastereomeric tuftsin analogues can be separated by means of hydrophobic ion-exchange chromatography using cross-linked polyelectrolytes known as Soloza K, these polyelectrolytes being copolymers of methaorylic acid, butyl methacrylate, and methylenebisacrylamide of varying composition. As may be seen from Fig. 2. the introduction of

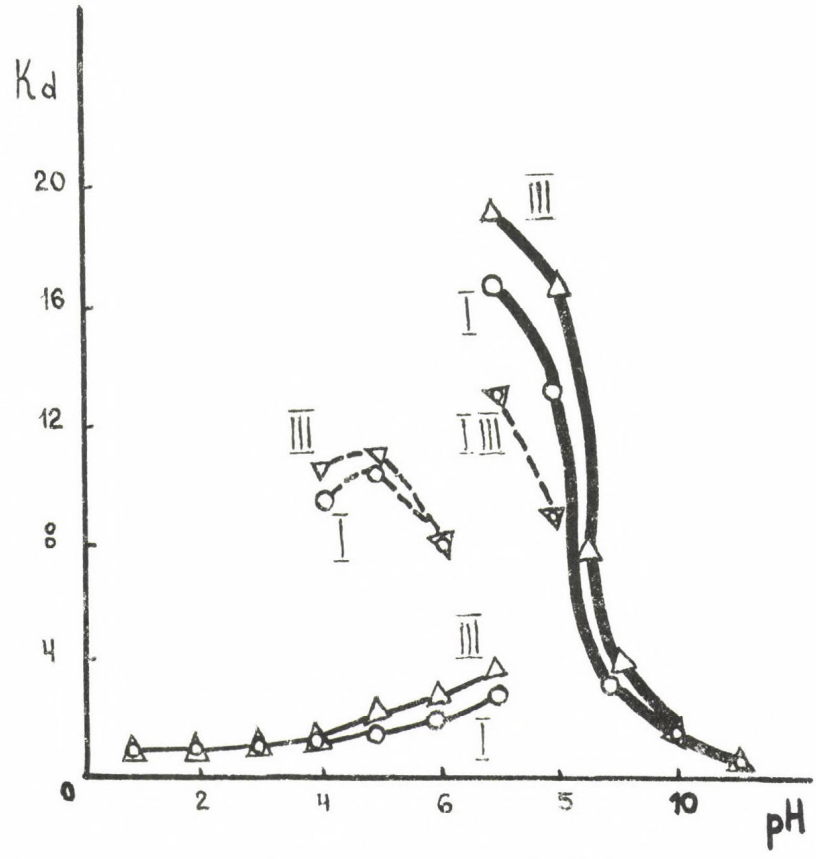

Fig. 4. Distribution coefficients (K_d) of tuftsin stereoisomers I and III versus the pH value of the buffer system used with the Soloza K 30/40 sorbent, and with carboxymethylcellulose (Sigma), pH range 1 to 7 (KCl + HCl, citric acid + Na_2HPO_4), pH range 8 to 11 (KCl + H_3BO_3 + NaOH)

carboxy groups into the hydrophobic copolymer of buthyl methacrylate and methylenebisacrylamide favours separation of diastereomers.

At the same time, the presence of the hydrophobic butyl methacrylate groupings in the Soloza K sorbent is apparently essential for the separation of the compounds under discussion as may be deduced from the data shown in Fig. 3 as well as from worse separation where the conventional carboxymethylcellulose

	ΔF° kcal/mole	ΔH° kcal/mole	ΔS° kcal/mole
LLLL	-1.46	-3.38	6.6
LLDL	-1.57	-3.48	6.5

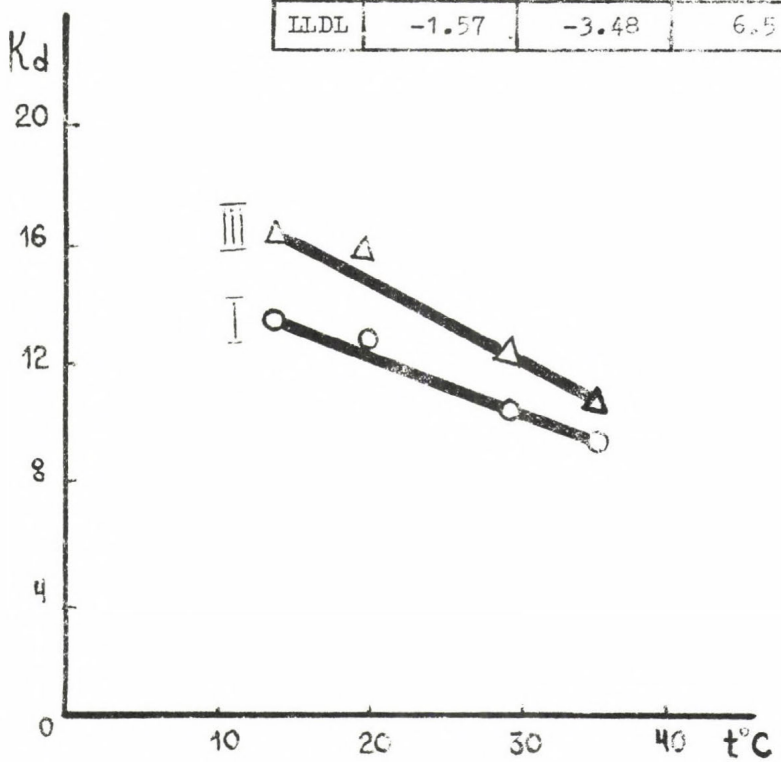

Fig. 5. Distribution coefficients (K_d) of tuftsin stereoisomers I and III versus temperature (t°C)
Sorbent: Soloza K 30/40
pH = 8.0, 0.1M borate buffer

ion exchanger is used (Fig. 4). Figure 4 also shows that the K_d of the diastereomeric analogues of tuftsin in the process of chromatography using the Soloza K sorbent, strongly depends upon the pH value and nature of the buffer system. Separation is best at pH = 8.0 in borate buffer. All diastereomeric impurities can be properly separated from tuftsin under such conditions (Table 1).

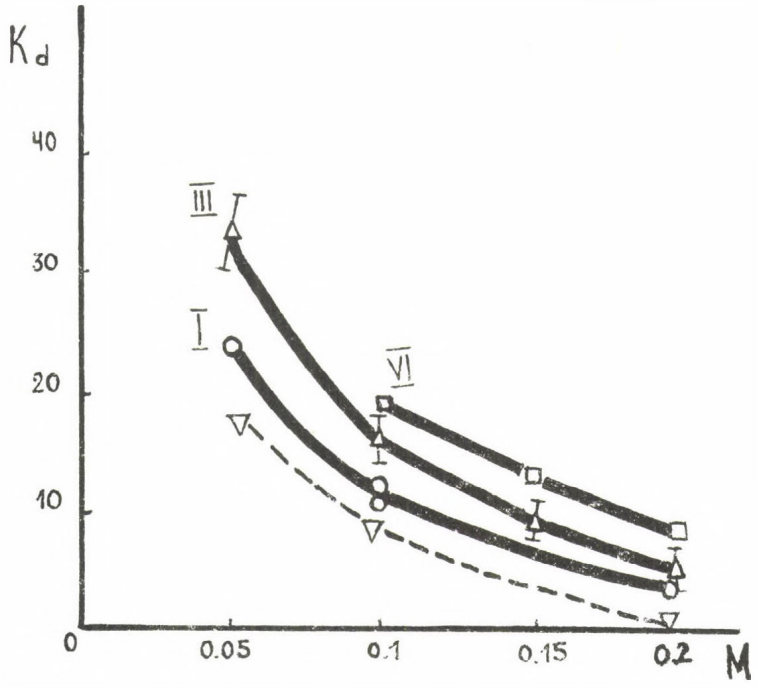

Fig. 6. Distribution coefficients (K_d) of tuftsin stereoisomers I and III and VI versus the molarity (M) of the buffer used with the Soloza K 30/40 sorbent, Soloza 20/40, and carboxymethylcellulose (Sigma), pH = 8.0, buffer: KCl + H_3BO_3 + NaOH

It will be interesting to note that the order of elution of diastereomeric tuftsin analogues in the process of chromatography using the Soloza K sorbent differs from the elution order incident to the high-performance liquid chromatography process. Evidently, the explanation lies in the fact that in the adsorption of diastereomeric tuftsin analogues by the Soloza K sorbent the major part is played by electrovalent forces rather than by hydrophobic reactions, as the case is in high-performance liquid chromatography, which fact is corroborated by the following data: (a) an obvious increase in K_d with increasing methacrylic acid content in the copolymer (Fig. 2), (b) a decrease in K_d with increasing temperature (Fig. 5), and (c) a sharp decrease in K_d with increasing ionic

force in the low ionic force region (Fig. 6). For all that, the hydrophobic reactions do play a certain part in the separation of diastereomeric peptides, as evidenced by the data contained in Figs 3 and 4 which we have discussed earlier.

Thus we have been able to show that hydrophobic ion-exchange chromatography can be used successfully, as well as high-performance liquid chromatography, for purifying synthetic diastereomeric derivatives of tuftsin.

The part that may be played by hydrophobic groupings in ion-exchange chromatography of diastereomeric peptides, using the Soloza K sorbent, requires further study.

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INTERMOLECULAR INTERACTION OF CARDIAC GLYCOSIDES, STEROID HORMONES AND CARBOHYDRATES IN LIQUID CHROMATOGRAPHY

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SUMMARY

The effect of the molecular structure of cardiac glycosides, steroid hormones and carbohydrates on their separation by high performance liquid chromatography was investigated.

For the separation of cardiac glycosides and steroid hormones silica support with bonded diphenylsilyl groups is very useful. Silica with adsorbed diamine molecules adsorbed from the eluent can be used for the separation and analysis of carbohydrates; from the diamines used as adsorption modifiers piperazine is the most convenient for the chromatographic analysis of carbohydrates.

Intermolecular interactions of the analyzed molecules on the adsorbent surface as well as with eluent molecules are considered.

The correlation of biological activity of cardiac glycosides with their retention on the hydrophobic surface of modified silica is discussed.

The difference in intermolecular interactions of separating substances with the adsorbent and eluent molecules and also interactions of the eluent molecules with the adsorbent and with each other is the basis separation by high-performance liquid chromatography. Since the concentration of the substances to be separated in solution is usually small, the intermolecular interactions of their molecules with each other do not have to be considered.

If the mass of adsorbent in a column, m , and specific surface area of adsorbent, s, are known then it is possible from the retention volume, V_R', to determine $V_g = V_R'/m$ and $V_s = V_g/s$. The retention volumes, V_R , are very convenient when we want to compare the retention on adsorbents differing only in specific surface area. The retention volume corresponding to the initial linear part of the adsorption isotherm is equal to the Henry constant of adsorption equilibrium, K_1. So by using chromatography it is easy to determine the thermodynamic adsorption characteristics such as Henry constant $K_{1\,g} = V_g$ (or $K_{1\,s} = V_s$) and, from its dependence on temperature, the differential enthalpy of adsorption $-\overline{\Delta H}_1$.

To demonstrate the effect of intermolecular interaction of the molecule with the surface functional groups of the adsorbent it is better to compare the thermodynamic adsorption characteristics such as the Henry constant, K_1 , or the retention volume, V_g , and the change of the differential enthalpy of adsorption $-\overline{\Delta H}_1$ of a given compound on silica with different bonded groups. Similar investigation has been made for silica with bonded hydrocarbon chains of different length [1] . The comparison of adsorbents with alkyl and phenyl groups is of great interest [2-5] .

The comparison of the ln V_g and $-\overline{\Delta H}_1$ values of cymarin on adsorbents with bonded groups having different carbon numbers is presented in Fig. 1. From Fig. 1 it can be seen that the ln V_g and $-\overline{\Delta H}_1$ values are approximately the same for number of carbon atoms from 6 to 18 in both the hydrocarbon and phenyl groups attached to the surface. For modifying groups having a smaller number of carbon atoms the ln V_g and

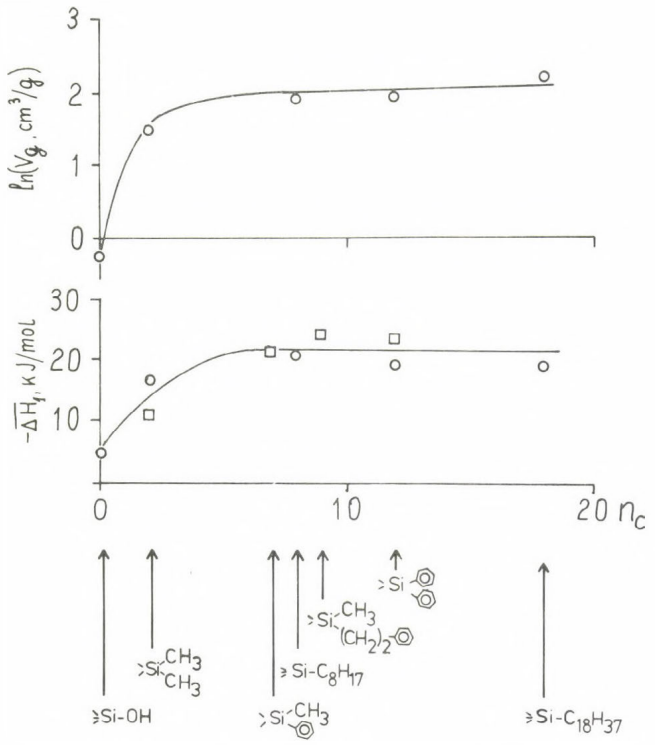

Fig. 1 Dependence of ln V_g and $-\overline{\Delta H}_1$ on the number of carbon atoms (n_c) on the groups bonded to the surface of silica gel. Sample: cymarin; eluent: 3:7 ethanol-water.

$-\overline{\Delta H}_1$ values decrease to the smallest values for adsorption on the non-modified, hydroxylated silica surface.

Thus for the analysis of cardiac glycosides and other substances separated by reversed-phase liquid chromatography, silica with bonded diphenylsilyl groups may be applied. Small differences in the retention on ODS packings as compared to packings with phenyl groups can be eliminated by changing the eluent composition. Separation of a cardiac glycoside mixture on silica with bonded diphenylsilyl groups is presented in Fig. 2 [3] and the separation of some estrogens on the same stationary phase is shown in Fig. 3 [6].

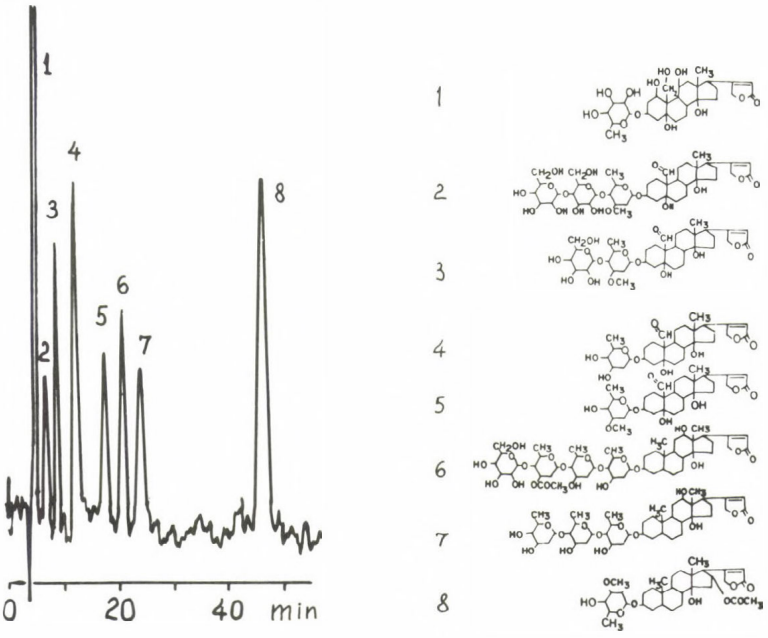

Fig. 2 Separation of cardiac glycosides on silica gel with bonded diphenylsilyl groups. Eluent: 2:3 ethanol-water. Temperature: 50°C. 1= G-strophanthin, 2= K-strophantoside, 3= K-strophanthin-β, 4= erysimin, 5= cymarin, 6= lanatoside C, 7= digoxin, 8= oleandrin.

In reversed-phase liquid chromatography the main intermolecular interactions determining separation are the Van der Waals interaction of molecules with hydrophobic groups and the strong intermolecular interaction of the eluent molecules with each other.

In the separation of carbohydrates another type of intermolecular interaction is used. In this case hydrogen bonding between the hydroxyl groups of the carbohydrates and the amino groups bonded to the surface of the stationary phase particles is used. For example, a number of publications deal with the separation and analysis of carbohydrates

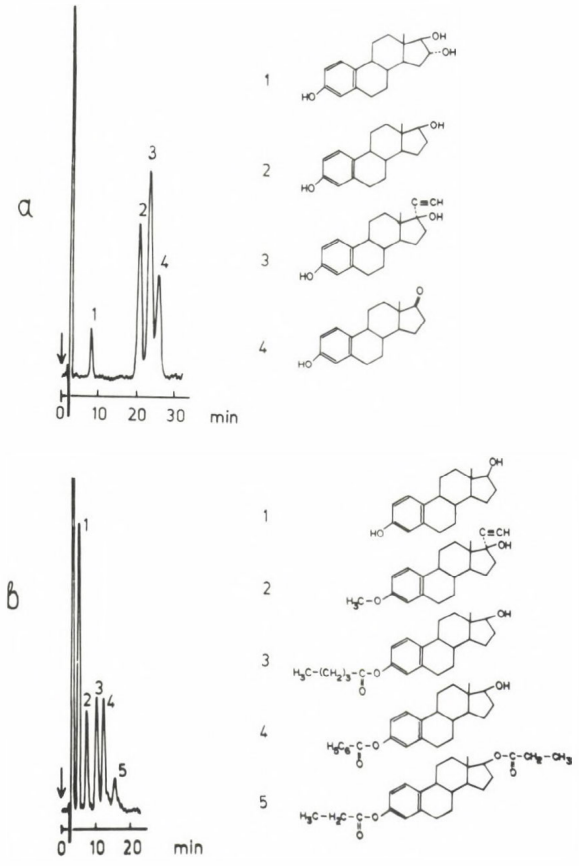

Fig. 3 Separation of estrogens hormones on silica gel with bonded diphenylsilyl groups at 50°C. Eluent: (a) 7:13 ethanol-water, (b) 3:2 ethanol-water. Peaks: (a) 1= estriol, 2= estradiol, 3= ethynylestradiol, 4= estrone; (b) 1= estradiol, 2= mestranol, 3= estradiol valerianate, 4= estradiol benzoate, 5= estradiol dipropionate.

on silica modified by γ-aminopropyltriethoxysilane [7,8]. In addition to chemical modification (bonding) modification of the silica surface by adsorption may also be used to form the layer of amino groups.

Fig. 4 presents models of the amino groups formed on the silica surface by bonded aminopropylsilyl groups or by adsorbed diamine molecules. On the adsorption of a diamine molecule, e.g., piperazine, one of the two amino groups produces a strong hydrogen bond with the silanol group while the second can interact with the proton-donor group of the carbohydrate molecule in the same way as the aminopropylsilyl group interacts with a sugar molecule.

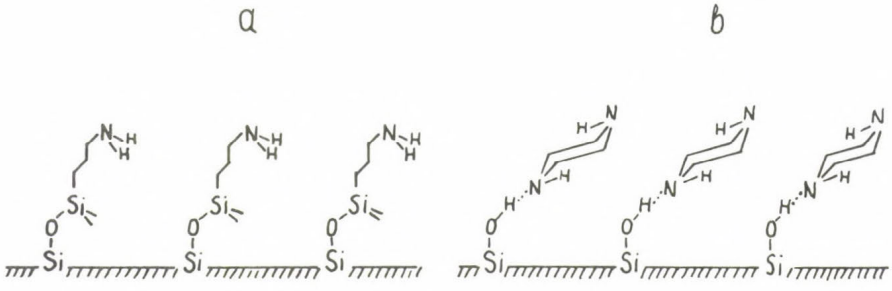

Fig. 4 Model of the modifying layer after (a) chemical and (b) adsorption modification.

Fig. 5 shows the separation of a sugar mixture on hydroxylated silica, on silica with piperazine molecules adsorbed from the eluent, and on bonded amino silica [9]. From this figure it can be seen that on a hydroxylated silica surface, using an eluent consisting of water and acetone, this mixture practically cannot be separated because the difference in the intermolecular interactions of the carbohydrates with the silanol groups from water solution is quite small. Moreover, the acetone and water molecules of the eluent are competitors forming quite strong hydrogen bonds with the silanol groups. After the addition of piperazine molecules to the eluent satisfactory separation of the sugar mixture takes place. The elution order of the carbohydrates is the following: monosaccharides elute first, then the disaccharides and finally

the trisaccharides, in accordance with the increasing number of proton-donor groups in their molecules. Similar separation of the same mixture takes place on silica with bonded amino groups.

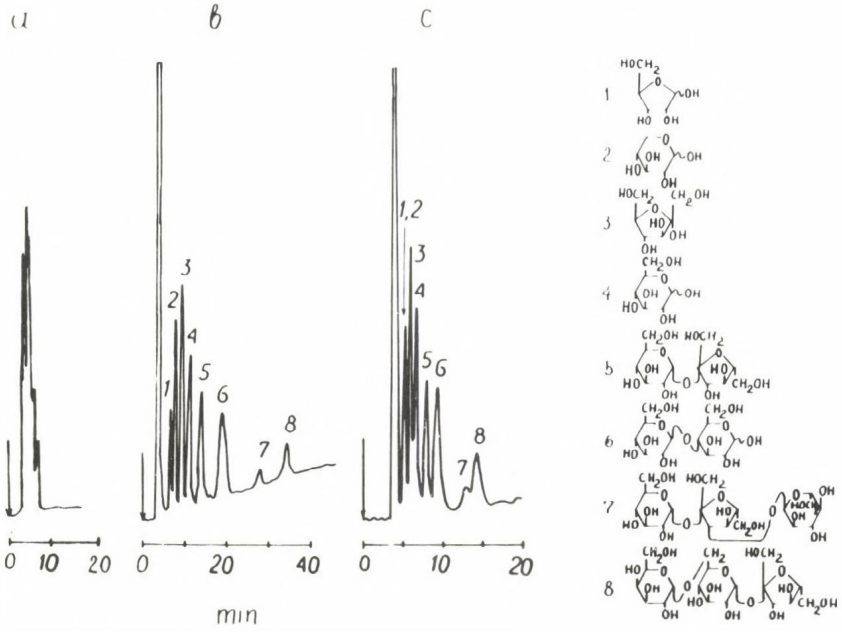

Fig. 5 Separation of carbohydrate mixture at 20°C from 4:1 acetone-water used as the eluent. Column packing: (a) hydroxylated silica gel; (b) the same silica gel with piperazine adsorbed from the eluent; (c) silica gel with bonded aminopropylsilyl groups. For (b) the eluent contained 0.44 mg/ml piperazine. Peaks: 1= ribose, 2= xylose, 3= fructose, 4= glucose, 5= sucrose, 6= cellobiose, 7= melezitose, 8= raffinose.

As shown, carbohydrates are separated due to hydrogen bonding with the electron-donor groups (amino groups) on the adsorbent surface. To increase the effect of this hydrogen bonding it is necessary to have a relatively large concentra-

tion of the electron-donor molecules in the eluent in order to decrease the adsorption of the water molecules on the surface amino groups. This is the reason why eluents consisting of water and acetonitrile or acetone are used for carbohydrate separation. Acetone is more available and cheaper and permits the detection of the carbohydrates by refractometry. The water content in the eluent is approximately 20% (by vol.).

In some recent papers [10 - 12] polyamines were described as adsorption modificators.

The effect of molecular geometry on retention is important; it is connected to the intermolecular interaction of the sample molecules with the adsorbent surface. Fig.6 shows the separation of some pentose and hexose stereoisomers.

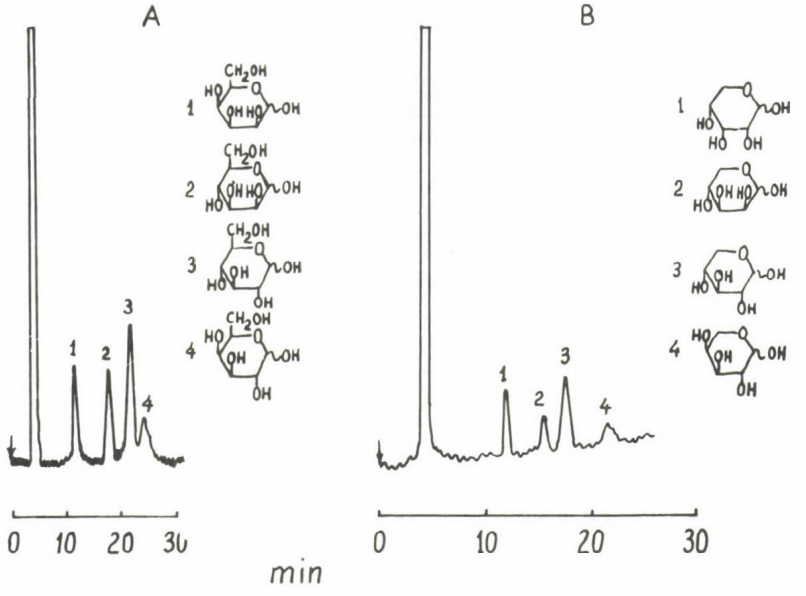

Fig. 6 Separation of (a) hexose and (b) pentose isomers on silica gel with piperazine adsorbed from the eluent. Eluent: (a) 17:3 and (b) 9:1 acetone-water, both including 0.44 mg/ml piperazine. Peaks: (a) 1= talose, 2= mannose, 3= glucose, 4= galactose; (b) 1= ribose, 2= lyxose, 3= xylose, 4= arabinose.

With help of the adsorption modificator piperazine it is possible to completely separate these isomers on hydroxylated silica. The elution order of mannose, glycose, galactose and lyxose, xylose, arabinose, is determined by the similarity in the geometry of their structure [9] . In spite of the fact that the number of hydroxyl groups in all pentoses is the same as well as in all hexoses, the different geometrical arrangement of hydroxyl groups in their molecules makes it possible to separate the isomers completely.

In the case of chromatography of cardiac glycosides the molecules of which contain aglycone and glycone (sugar part), the intermolecular interactions are more complicated. First, it is possible to note the effect of the geometry of the molecules on their separation. Fig. 7 shows the separation of desglucoheirotoxin and convallatoxin [4] . These molecules are identical from the point of view of their general formulae but the same aglycon-strophantidin is connected to α-L-rhamnose (convallatoxin) and β-D-gulomethylose (desglucoheirotoxin). Such a difference results in various configurations of the whole molecule. As a conclusion, the more flat

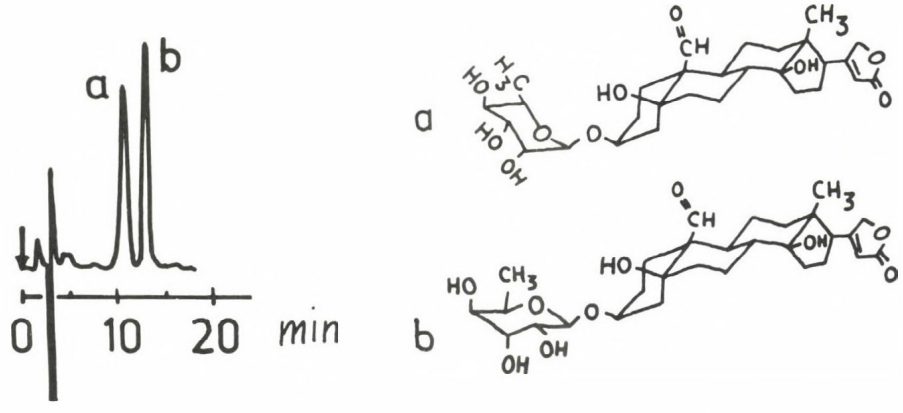

Fig.7 Separation of (a) convallatoxin and (b) desglucoheirotoxin on silica gel with bonded diphenylsilyl groups from 3:7 ethanol-water used as the eluent. Temperature: 40°C.

desglucoheirotoxin molecule is retained stronger than the molecule of convallatoxin. In this case the difference in the main dipole moment of the glycoside molecules surrounded by a polar eluent and the possibility of arranging more contact with the adsorbent surface are the important forces.

We have tried to evaluate the role of glycone and aglycone in the intermolecular interaction of cardiac glycosides during their separation on silica with bonded diphenylsilyl groups. Fig. 8 [4] shows examples of cardiac glycoside separation. The glycoside mixtures were chosen in such a manner that the glycones were similar in size in each compound group. This figure illustrates that the aglycone structure, the number of hydrophilic and hydrophobic groups and the position of these groups in the aglycones have a great influence on the retention of the cardiac glycosides. For example, in Fig. 8a all compounds may be subdivided into three types of glycosides: glycoside with the most hydrophilic oubagenin-aglycone, glycosides with strophanthidin aglycone and glycoside with oleandrigenin aglycone. The effect of the properties of the aglycone on the retention of the glycoside is shown most clearly in the separation of lanatosides A,B and C (Fig. 8d), having the same glycone. The most hydrophobic aglycone in these samples is digitoxigenin; therefore, lanatoside A has the longest retention time. Introduction of a hydroxyl group in C_{16} position decreases by more than a factor of two the retention time of lanatoside B while introduction of a hydroxyl group to position C_{12} decreases the retention time of lanatoside C by a factor of five in comparison to lanatoside A. Fig. 8 also demonstrates that glycosides with the same aglycone are separated according to the hydrophilic and hydrophobic properties of the glycones.

To compare the intermolecular interaction of the investigated cardiac glycosides we have measured their retention volumes on silica with bonded diphenylsilyl groups keeping the eluent composition and the temperature constant. The glycosides were arranged according to the increasing hydrophobic properties of their aglycon, and glycosides with the same aglycone were arranged according to the increasing hydrophobic

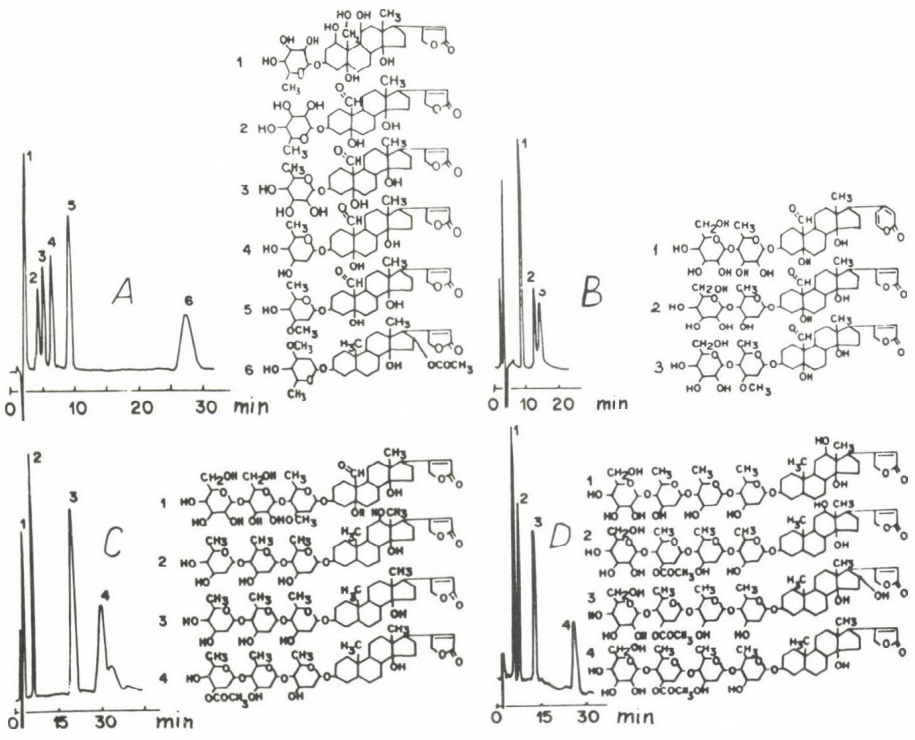

Fig. 8 Separation of cardiac glycoside mixtures on silica gel with bonded diphenylsilyl groups. Eluent: ethanol-water in the following proportions: (a) 7:13, (b) 3:7, (c) and (d) 2:3. Temperature: (a) and (d) 50°C, (b) 40°C, (c) 60°C. Peaks: (a) 1= G-strophanthin, 2= convallatoxin, 3= desglucoheirotoxin, 4= erysimin, 5= cymarin, 6= oleandrin; (b) 1= corelborin-π, 2= olitoriside, 3= K-strophanthin-β; (c) 1= K-strophanthoside, 2= digoxin, 3= digitoxin, 4= acetyldigitoxin; (d) 1= desacetyllanatoside C, 2= lanatoside C, 3= lanatoside B, 4= lanatoside A.

properties of their aglycon. Fig.9 presents a diagram from which it is clear that the retention of cardiac glycosides is determined by the relative number of the hydrophilic and hydrophobic groups in their molecule. Compounds in Fig. 9 are: 1= G-strophanthin, 2= K-strophanthoside, 3= corelborin ,

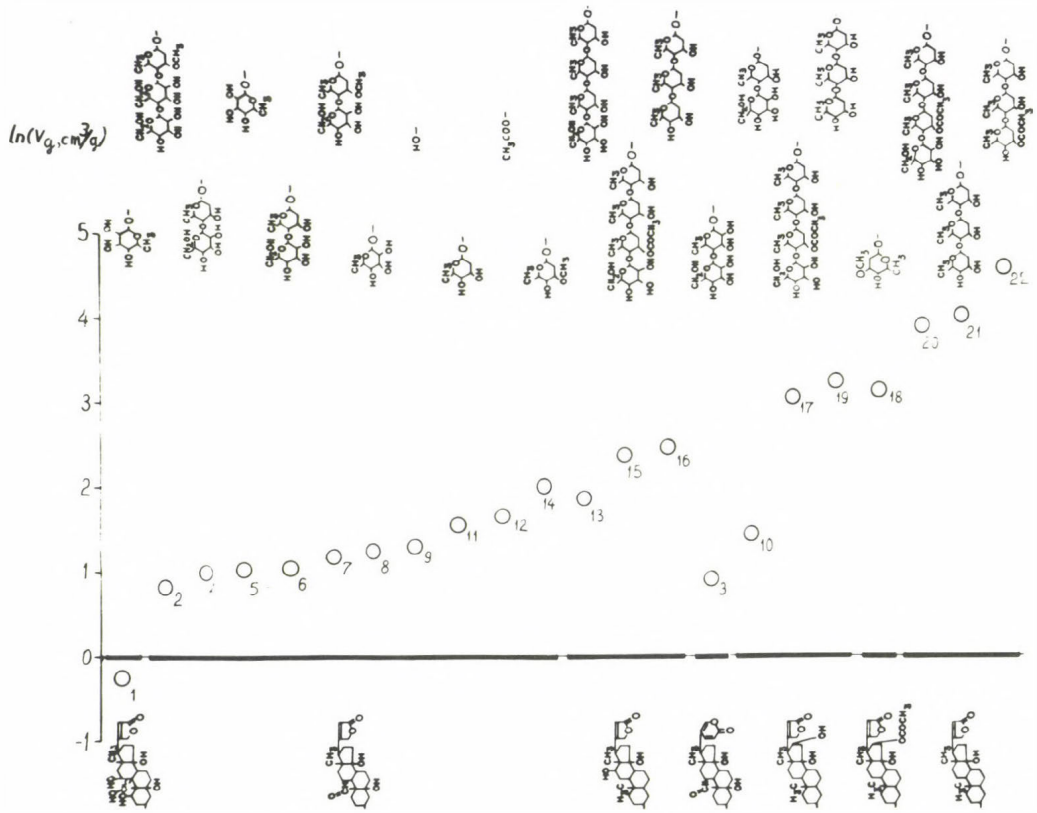

Fig. 9 Correlation of the retention (ln V_g) of cardiac glycosides on silica gel (LiChrosorb SI 60) with bonded diphenylsilyl groups, with increasing hydrophobic properties of the aglycone and glycone. Temperature: 50°C.

4= erysimoside, 5= convallatoxin, 6= olitoriside, 7= K-strophanthin-β, 8= desglucoheirotoxin, 9= strophanthidin, 10= glucogitoroside, 11= erysimin, 12= strophanthidin acetate, 13= desacetyllanatoside C, 14= cymarin, 15= lanatoside C, 16= digoxin, 17= lanatoside B, 18= oleandrin, 19= gitoxin, 20= lanatoside A, 21= digitoxin, 22= acetyldigitoxin. Although the contribution of the aglycone to the retention is significant, the role of the glycone is very important in determining the retention.

It is important to investigate the effect of the number of monosaccharide groups in the glycone of the cardiac glycosides on their retention. Fig. 10 presents the relationship

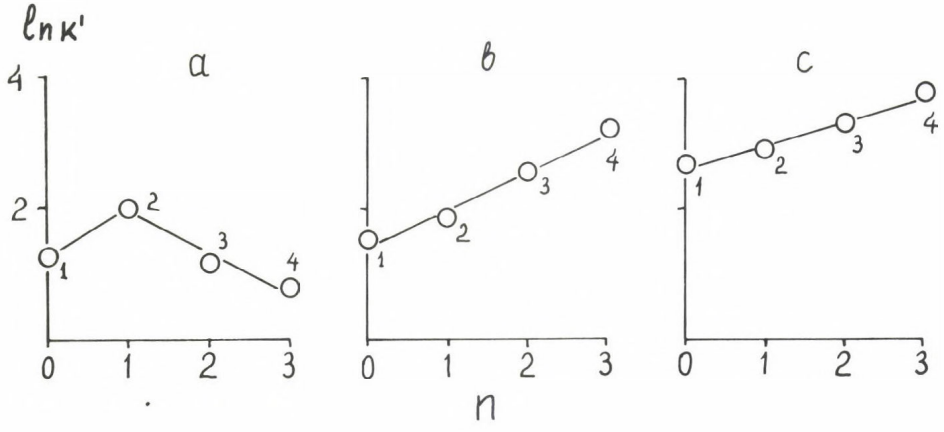

Fig. 10 Dependence of ln k' of the cardiac glycosides on the number of monosaccharide groups in their glycone. Eluent: (a) 7:13 ethanol-water, (b) 1:3 acetonitrile-water, (c) 1:2 acetonitrile-water. Adsorbent: (a) silica gel with bonded diphenyl groups, (b) and (c) silica gel with bonded octadecylsilyl groups. Peaks: (a) 1= strophanthidin, 2= cymarin, 3= K-strophanthin-β, 4= K-strophanthoside; (b) 1= digoxigenin, 2= digoxigenin monodigitoxoside, 3= digoxigenin bisdigitoxoside, 4= digoxin. Results presented in (b) and (c) were obtained using the data of Castle [13].

between the capacity factors of strophanthidin, digoxigenin, digitoxigenin and their derivatives and the number of monosaccharide groups in their molecules.

The data for digitalis glycosides were evaluated based on the results published by Castle [13]. It is seen from Fig.10 that, in the case of the more hydrophilic monosaccharide (glucose) increasing their number results in a decreasing retention of the cardiac glycosides on the hydrophobic surface. On the other hand, increasing the number of the less hydrophilic monosaccharide (digitoxose) in the glycone results in an increase in the retention. In spite of the fact that the correlations were obtained on different adsorbents and from eluents with different compositions, the general trend will probably be similar for any hydrophobic adsorbent.

It is necessary to note that similar correlations also exist in other systems and not only in reversed-phase chromatography. Fig. 11 shows a quite similar dependence of the

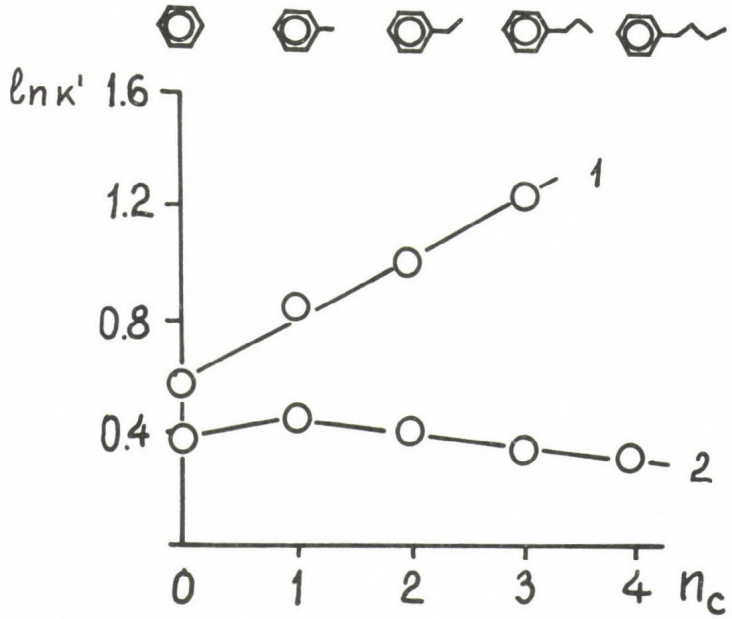

Fig. 11. Dependence of ln k' of alkylbenzenes on the number of carbon atoms in the hydrocarbon chain. Eluent:(1) water,(2) n-hexane. Adsorbent: hydroxylated silica gel. Temperature: 20°C.

retention of benzene and its alkyl derivatives on silica from hexane and water [5]. In this case, the dependence of the retention on the number of carbon atoms in the hydrocarbon chain is due to the different polarity of the eluent while in the previous case the dependence was due to the change of the polarity of the groups in the glycone part at similar eluent polarities.

Fig. 12 shows the separation of cardiac glycosides with different lengths of the glycone parts.

In the case of steroid hormones, it is also possible to arrange them according to their increasing hydrophobic properties [6]. Fig. 13 presents the correlation between the retention volume and the hydrophobic properties of steroid molecules. Compounds in Fig. 13 are: 1= prednisolone, 2= hydrocortisone, 3= estriol, 4= prednisone, 5= cortisone, 6= androsterone, 7= corticosterone, 8= prednisone acetate, 9= cortisone acetate, 10= estradiol, 11= testosterone, 12= ethynylestradiol, 13= methandrostenolone, 14= pregnin, 15= methylestradiol, 16= estrone, 17= methyltestosterone, 18= progesterone, 19= desoxycorticosterone acetate, 20= mestranol, 21= megestrol acetate. Here again, retention is determined not by the number of hydrophobic or hydrophilic groups in the molecules but by the relative quantity of these groups.

It is possible to assume that the molecule consists of different groups and each group brings its own contribution to the retention of the molecule. A similar assumption for different atoms in a molecule is accepted in chromatoscopic investigations developed by Kiselev and coworkers [14-16].

Thus $\ln V_g = \sum a_i n_i$ where n_i is the number of functional groups in a molecule and $a_i = \ln(V_g)_i$ is the retention volume of the i-th group. Similarly $\ln V_s = \sum a_i n_i$ and in this case $a_i = \ln(V_s)_i$.

In order to evaluate the average contribution of the functional groups to the retention, one may consider as the first approximation that all similar groups have the same contribution. This is not exactly true, for example, it was already shown that hydroxyl groups change the retention of

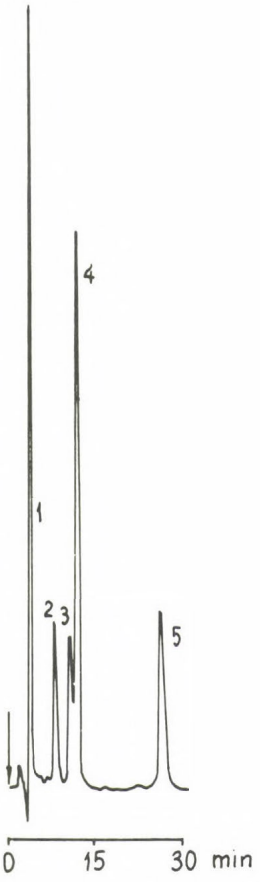

Fig. 12 Separation of cardiac glycosides on silica gel with bonded ODS groups. Eluent: 3:7 ethanol-water. Peaks: 1= G-strophanthin, 2= K-strophanthoside, 3= K-strophanthin-β, 4= strophanthidin, 5= cymarin.

glycosides differently according to their position in the aglycone. The more number of molecules are used for the evaluation of the average contribution the easier it will be to determine its value. For this purpose we have selected the molecules of cardiac glycosides and steroid hormones having

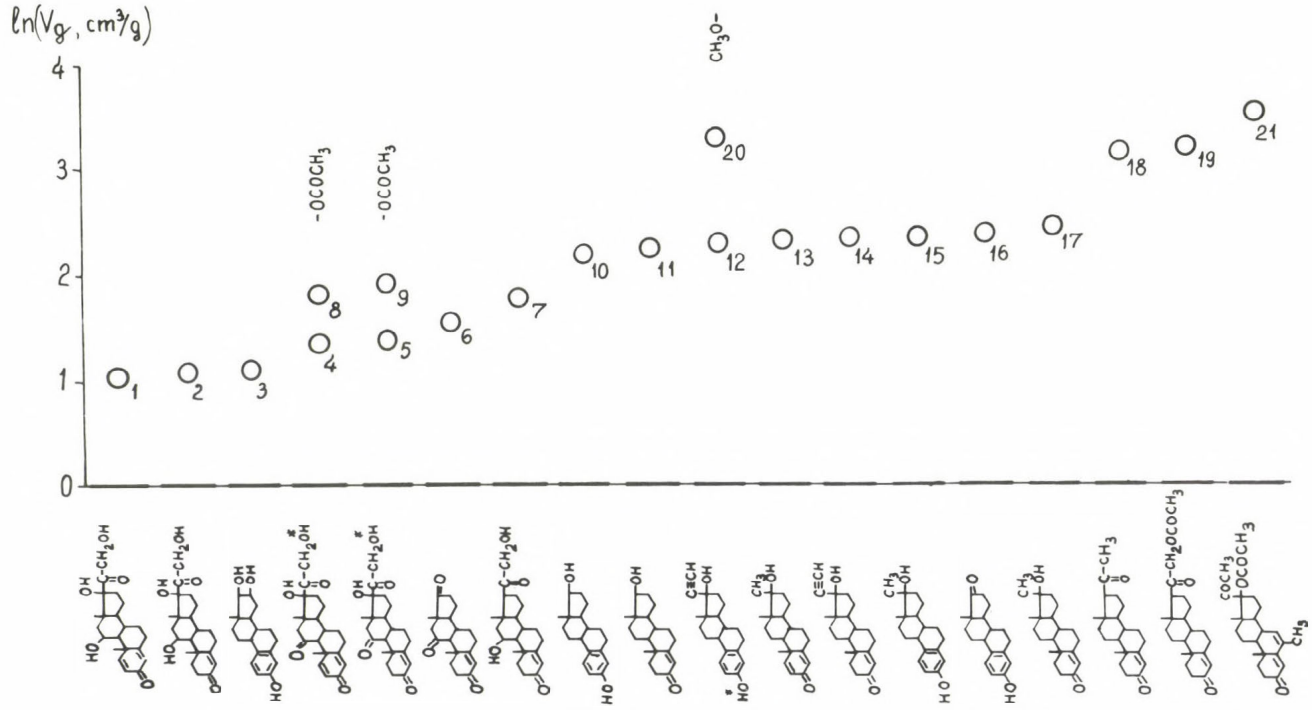

Fig.13 Correlation of the retention (ln V_g) of steroid hormones on silica gel (LiChrosorb SI 100) with bonded diphenylsilyl groups, with increasing hydrophobic properties of molecules. Eluent: 7:13 ethanol-water. Temperature: 50°C.

many common structures. Let us assume that these molecules consist only of the following groups:

$$-OH,\quad -O-,\quad H-\overset{|}{C}=O,\quad -\overset{|}{C}=O \quad \text{and} \quad CH_n -$$

(n = 0,1,2,3). Let us also assume that the contribution of the following groups are the same:

$$-\overset{|}{\underset{|}{C}}-,\quad -C\equiv,\quad -\overset{|}{C}=,\quad -\overset{|}{C}H,\quad -CH_2-,\quad -CH_3,\quad =CH-,\quad \equiv CH$$

Strictly speaking this is not true because from the point of the intermolecular interaction, the properties of these groups is not exactly the same. Nevertheless, for the sake of simplicity, such an approximation is accepted.

In order to evaluate the retention of a molecule ($\ln V_g$) at standard conditions it is necessary to summarize the contributions of a number of groups on retention. In order to determine these contributions it is necessary to solve the system of linear equations by the least squares method.

Since the retention volumes of cardiac glycosides and steroid hormones were determined under the same conditions but on adsorbents with different specific surface area, it was necessary to calculate the V_s values. The degree of modification of the silica gels LiChrosorb SI 100 and LiChrosorb SI 60 which were used for the determination of the retention volumes of steroid hormones and cardiac glycosides is similar [3]. By using the comparable retention volumes, V_s, of 22 cardiac glycosides and 21 steroid hormones [6] the contribution of the different functional groups to retention were calculated. The results are presented in Table 1.

Fig. 14 shows the correlation between the experimentally determined retention volume values ($\ln V_s$) and those calculated from the number of each groups and their relative contribution to retention. As can be seen, the calculated and experimental values coincide quite well. The retention volumes ($\ln V_s$) of aglycones of the investigated glycosides were calculated on the basis of data in Table 1 and are presented in Table 2.

Table 1. Values of a_i for different functional groups of cardiac glycosides and steroid hormones at standard conditions*.

Functional group	a_i
$-OH_{(C12)}$	- 1.4603
$-OH_{(C16)}$	- 0.8188
- OH	- 0.6055
-O-	- 0.2315
-C=O	- 0.1598
H-C=O	- 0.7854
CH_n	+ 0.2444

Approximation

It is assumed that all OH groups except $OH_{(C12)}$ and $OH_{(C16)}$ of the cardiac glycosides and steroid hormone molecules are identical. Groups $-\overset{|}{\underset{|}{C}}-$, $-\overset{|}{C}=$, $-C\equiv$, $-CH_3$, $-\overset{|}{CH}_2$, $-\overset{|}{\underset{|}{CH}}$, $=\overset{|}{CH}$, $\equiv CH$ are considered as identical also.

*Adsorbent: silica gel with bonded diphenylsilyl groups; eluent: 7:13 ethanol-water; temperature: 50°C. Values of a_i are determined from the following equation: $\ln V_s = \Sigma a_i n_i$.

Evaluation of the contribution of the aglycone to the retention of cardiac glycosides by means of coefficients corresponding to the various functional groups shows that the arrangement of the glycosides according to the increasing hydrophobic properties of their aglycone (Fig. 9) was correct.

The calculated values of the retention volumes of digoxigenin, gitoxigenin and digitoxigenin reflect the increasing capacity factors (ln k') of these compounds as determined by Erni and Frei [17] .

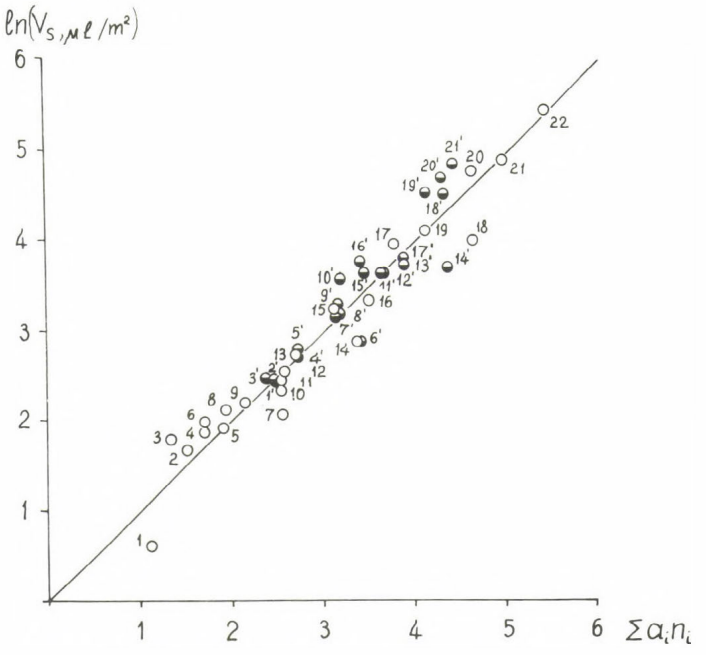

Fig. 14 Correlation of the experimentally determined retention volumes (ln V_g) of cardiac glycosides and steroid hormones and the values calculated according to the additivity using the coefficients of Table 1. Open circles represent cardiac glycosides (the numbers correspond to the compounds in Fig. 9), while semiclosed circles represent steroid hormones (the numbers correspond to the compounds in Fig. 13).

It is interesting to consider the correlation between the biological activity and the retention volumes of cardiac glycosides on the hydrophobic surface of silica. It is more proper to express the biological activity (LD - lethal dose) of cardiac glycosides by μmole per unit of the weight of the cat used for testing (μmol/kg) than by mg/kg [18].

Fig. 15 shows the correlation between the biological activity and the retention volumes (ln V_g) of the cardiac glycosides on silica with bonded diphenylsilyl groups. From this figure it is clear that with increasing retention volumes

Table 2. Calculated values of $\ln V_s = \Sigma a_i n_i$ at standard conditions (see Table 1) for aglycones of cardiac glycosides.

Aglycone	$\ln V_s$
Oubagenin	1.3525
Strophanthidin	2.1392
Digoxigenin	2.3142
Gellebrigenin	2.3836
Gitoxigenin	2.9557
Oleandrigenin	3.6276
Digitoxigenin	3.7745

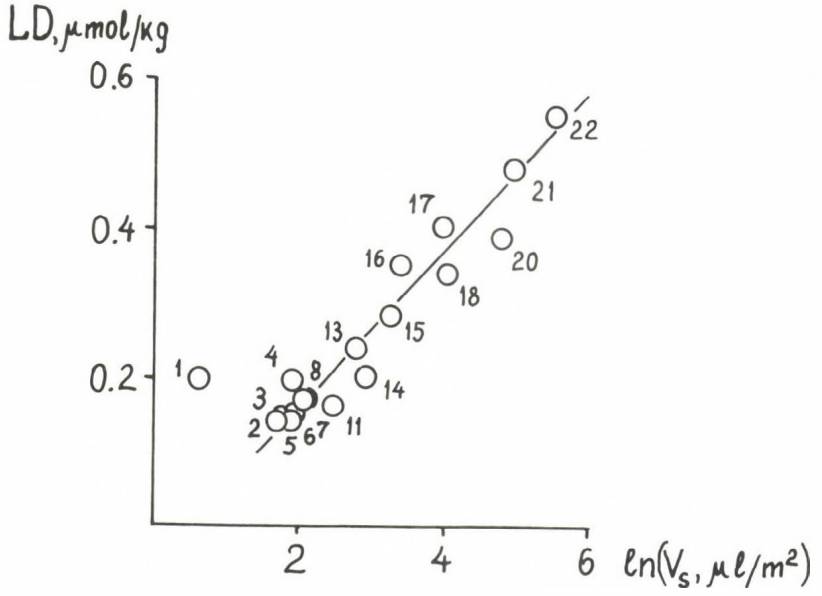

Fig. 15 Correlation of the biological activity (LD) of cardiac glycosides with their retention volume ($\ln V_s$) on silica gel with bonded diphenylsilyl groups.

of the cardiac glycosides their biological activity decreases. This correlation shows that the biological activity of cardiac glycosides is connected with hydrophilic and hydrophobic properties of their molecules which determine the transport glycosides to the receptors.

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GAS CHROMATOGRAPHIC ANALYSIS OF AMITRIPTYLINE IN THE CENTRAL NERVOUS SYSTEM OF RATS

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INTRODUCTION

During the past decade efforts have been made to elucidate the kinetics of amitriptyline (AMI), an antidepressant agent of widespread clinical use /1/, and to apply this kinetic knowledge to the treatment of depression or to the explanation of results of pharmacological experiments. However, these studies have been limited only to the plasma level/effect relationship of AMI, and no attention has been paid to the relation between the drug concentration in its target organ, i.e., the central nervous system (CNS) of experimental animals and its pharmacological response.

We have found previously /2, 3/ that the distribution of another tricyclic antidepressant, imipramine, was uneven in rats, and that its pharmacokinetics in blood markedly differed from that in the rat CNS. In addition, it has been also found that prolonged administration of imipramine to rats inhibited its elimination from blood, and this in turn produced a cumulation of the drug and its main, active metabolite desipramine, in the peripheral compartment, particularly in the rat brain /2, 3/. Therefore, we have carried out a more detailed study of the pharmacokinetics of AMI in rats, simultaneously assaying the drug level in the blood plasma and in the CNS, after single and multiple dose of AMI. As prolonged administration of AMI to experimental animals simulates the clinical practice (an improvement of depressive patients has been observed after long-term treatment with AMI or other antidepressants), it is used

as a standard dosage schedule in all pharmacological experiments in our Institute with antidepressant agents.

METHODS

Experiments were carried out on male Wistar rats (180-220 g) fed with the standard granulated diet (Bacutil). Animals were deprived of food for 24 h before experiments, but they had free access to tap water.

Amitriptyline (AMI) (hydrochloride, Natterman, Köln) was given to rats as a solution in bidistilled water, in a single or multiple (for 14 days at 24 h intervals) dose of 10 or 20 mg/kg i.p. The animals were killed at different times after AMI administration, the blood was collected to heparinized tubes, the whole brain or cortex and spinal cord were dissected and stored on solid CO_2 until assessment, but not longer than 18 h. Preparation of samples and extraction procedure were performed according to Burch et al. /4/.

Control animals received bidistilled water in the same dosage schedule as the experimental groups.

Chromatography

Pye-Unicam GC system fitted with a flame ionization detector and glass column (1 m x 4 mm) was used for assay procedure. The column was packed with 10% OV-17 on Gas-Chrom Q (80-100 mesh). Temperature: injector and oven = 230°C, detector = 270°C. Gas flow: N_2 = 75 ml/min, H_2 = 50 ml/min, air = 300 ml/min. All injections were of 10 µl volume. Retention time of AMI = 5.6 sec, sensitivity = 500 ng/sample, recovery = 75%. The method did not allow to assay the main metabolite of AMI nortriptyline, as the level of the latter was below the limit of detection.

RESULTS

As indicated in figures 1A and 1B no interferences exist between the sample and blank chromatograms, as well as between the peaks of AMI and nortriptyline.

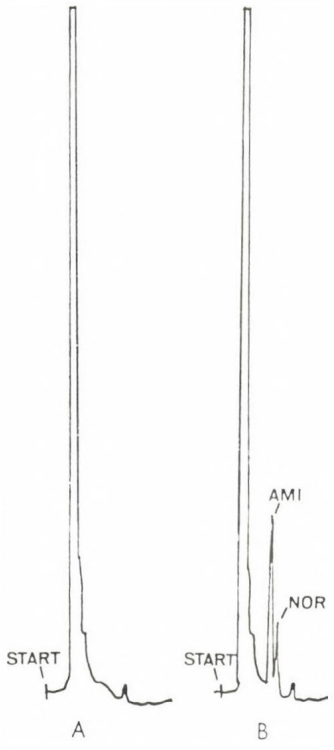

Fig. 1A - Chromatogram from 2 ml blank of brain homogenate carried through the extraction procedure
1B - Chromatogram from 2 ml of brain homogenate with internal standard of AMI (10 µg) and nortriptyline (10 µg)

After a single dose of 20 mg/kg i.p., AMI rapidly penetrated into the blood and brain of the rats (t_{max} for both = 30 min), but its brain level exceeded in statistically significant manner the corresponding level in blood plasma. The rate of elimination of the drug from blood and brain was similar and 10 h

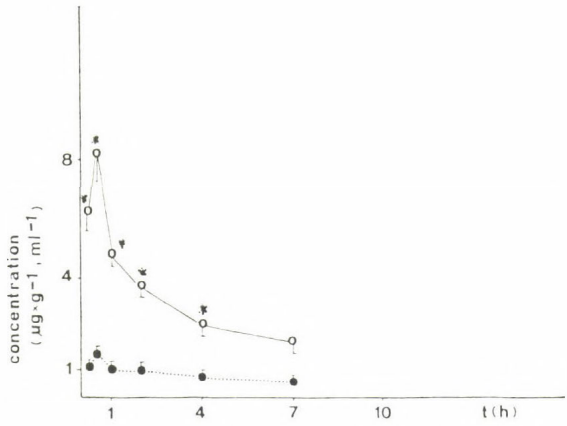

Fig. 2. Blood ●...● and brain o___o concentration of AMI at different times after AMI administration in a single dose of 20 mg/kg i.p. Each point is the mean of 5-7 animals ± SEM. * $p<0.01$ when compared with the corresponding blood level (Student t-test)

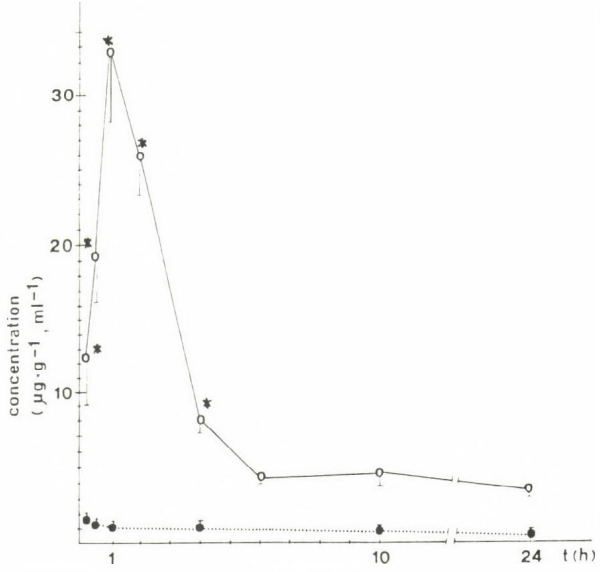

Fig. 3. Blood ●----● and brain o___o concentration of AMI at different times after prolonged administration of the drug, 14 x 20 mg/kg i.p. Each point is the mean of 5-7 animals ± SEM. * $p<0.01$ when compared with the corresponding blood level (Student t-test)

after administration the concentration of AMI in these tissues were below the limit of detection (Fig. 2).

Prolonged administration of AMI to rats (14 x 20 mg/kg i.p.) produced significant inhibition of its elimination from both blood and brain, and 24 h after the last dose the drug was still present in the blood and brain. Time/concentration curve of blood, after short lasting (approx. 4 h) period of an increase of AMI level was nearly flat, indicating that steady-state was achieved (Fig. 3).

Cortex/spinal cord ratios for AMI concentrations, after single (10 mg/kg i.p.) or multiple (14 x 10 mg/kg i.p.) dose treatment, was nearly the same, 2.3 and 2.4 respectively. This indicated that the distribution of AMI within the CNS of rats was not uniform and that these ratios among specific areas of CNS were stable and did not change after repeated treatment (Fig. 4).

Pharmacokinetic data presented in Table 1 show that the rates of elimination of AMI from blood and brain differed slightly (faster elimination of the drug from the brain);

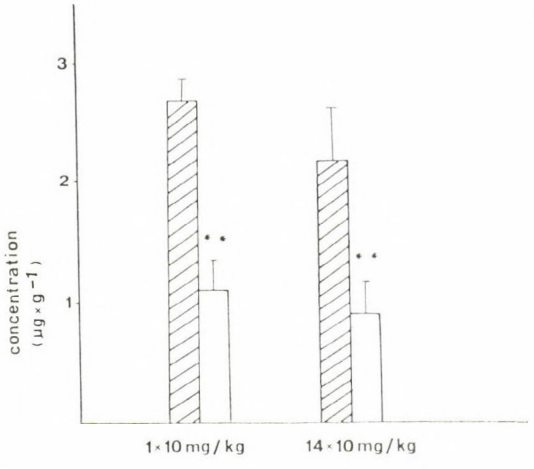

Fig. 4. Cortex ▨ and spinal cord □ level of AMI 1 h after administration of the drug in a single, 10 mg/kg i.p. or multiple, 14 x 10 mg/kg i.p. dose. The results are presented as the mean of 5 animals ± SEM. ** $p<0.01$ when compared with the corresponding level in the cortex

Table 1.

Pharmacokinetic parameters of AMI calculated from blood and brain data, after single dose (20 mg/kg i.p.) or prolonged (14 x 20 mg/kg) administration of AMI.

	1 x 10 mg/kg i.p.		14 x 20 mg/kg i.p.	
	blood	brain	blood	brain
$t_{0.5}$ (h)	5.50	4.74	25.60	18.23
AUC ($\mu g.h.ml^{-1}$ or g^{-1})	8.73±1.20	45.20±5.02[a]	29.62±2.70[b]	294.73±32.5[b]
Cl_t (l/h)	0.45 (kg/h	0.08[c]	0.13	0.01[c] (kg/h)

a - $p<0.01$ compared to the corresponding value of blood;
b - $p<0.001$ compared to the corresponding values obtained after single dose of AMI (Student t-test);
c - "purification coefficient" calculated as dose/AUC brain.

however, the differences did not reach the statistically significant level. Prolonged administration of AMI inhibited elimination of the drug from both blood and brain, and this produced a statistically significant increase of the corresponding AUC values. As the brain concentration of AMI was much higher than that in blood and the rates of elimination from blood and brain did not significantly differ, the blood purification of AMI, expressed as the total body clearance (Cl_t) proceeded approx. 5 times (after single dose) or 13 times (after multiple dose) faster than the purification of the brain tissue.

DISCUSSION

The presented results indicate that similarly to other antidepressant drugs such as imipramine, desipramine and citalopram /2, 3, 5/, AMI cumulates in the rat brain: its level in the tissue exceeded, in statistically significant manner, the cor-

responding blood level, particularly within the first 4 h after administration of the drug, i.e., at the time when the standard behavioral observations are performed in pharmacological experiments. These results are in agreement with the previous results of Jørgensen et al. /6/ who found that the brain/blood ratios for AMI concentration at different times after AMI administration decreased over the time of the first 4 h from 7.0 to 2.0 (these data were obtained after i.v. administration of AMI).

Prolonged administration of AMI to rats produced an inhibition of its elimination from blood and brain. Consequently 24 h after administration of the last dose, AMI was still present in both tissues and, in addition, its blood and brain concentration at this time exceeded the IC_{50} values of AMI for inhibition of neuronal uptake of noradrenaline and serotonin /7/ which are regarded as markers of its antidepressive activity. Prolongation of the biological half-life of AMI after repeated treatment may support the suggestion of Ziegler et al. /8/ to use a single daily dose of AMI in clinical practice and also in pharmacological experiments because, after multiple dose, the $t_{0.5}$ value of AMI increased up to 25 h.

Our results also indicate that the distribution of AMI within the CNS of rats is not uniform. There are differences between the cortex and spinal cord level of the drug at the same time after administration. It is difficult to say at this moment what does this mean for the pharmacological effects of AMI. However, as results of the previous binding study carried out after prolonged administration of antidepressants or after prolonged treatment with electroconvulsive shock /9, 10/ indicate, adaptative changes within the rat brain in the number of different receptors appear in some specific brain areas and do not exist in others. Therefore, it is possible that these differences may be induced, at least partially, by the differences of actual concentration of the antidepressants in the investigated part of rat CNS.

The presented results may have a more general meaning for pharmacokinetic/pharmacological study. Any correlation between pharmacokinetics of centrally acting drugs and their pharmaco-

logical response should also take into account their concentration in the CNS of experimental animals, as pharmacokinetics of these drugs in blood may not reflect their distribution in the target organ. Consequently, any conclusion driven from pharmacokinetic blood data may be completely unvalid.

SUMMARY

Pharmacokinetic study of amitriptyline (AMI) was carried out in rats after single and multiple dose treatment with the drug. AMI concentration in blood plasma, brain, frontal cortex and spinal cord was assayed using gas chromatography. It was found that AMI distribution in rats was uneven: its level in the brain tissue was higher than the corresponding level in the blood plasma. Also, within the central nervous system of rats AMI was not distributed uniformly. Prolonged administration of the drug produced an inhibition of AMI elimination from blood and brain and cumulation of the drug within the peripheral compartment.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF VINCA ALKALOIDS

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SUMMARY

Separation of Vinca alkaloids using reversed-phase high-performance liquid chromatography was investigated on Bondapack C_{18} and Polygosil C_{18} columns with acetonitrile-aqueous 0.01 M ammonium carbonate mixture as the eluent. This method could be successfully applied for solving different problems. Plant extracts as well as reaction mixtures of partial synthesis starting from indole alkaloids have been analysed by this method.

INTRODUCTION

Earlier in our laboratory we isolated some Vinca alkaloids from different parts of Amsonia tabernaemontana /1-6/ cultivated in Hungary. Tabersonine was found to be the major alkaloid. Up till now tabersonine as well as minor alkaloids isolated from plants were identified by means of thin-layer chromatography, spectrophotometry and by their physical constants. The application of high-performance liquid chromatography seemed to provide a rapid and simple method for the qualitative and quantitative analysis of these alkaloids.

The major alkaloid of Amsonia tabernaemontana, tabersonine, can be used as the starting material of the very important partial synthesis of the Vinca alkaloid, vincamine /7-10/. With the help of HPLC we could determine the optimal conditions

of the partial synthesis starting from tabersonine or hydrogenated tabersonine.

EXPERIMENTAL

Liquid chromatography was performed using a Waters Model ALC/GPC 201 chromatograph equipped with an U6K injector and a variable-wavelength Waters Model 440 UV Detector.

The separations were performed on reversed-phase columns packed with Bondapack C_{18}/Corasil (610 x 2 mm I.D.) (Waters Assoc., Frankfurt, FRG) and Polygosil 60-10 C_{18} (250 x 4.5 mm) (Macherey-Nagel Co., Düren, FRG).

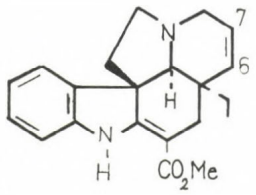
The chromatographic solvents were mixtures of acetonitrile (HPLC Grade, Fischer Scientific Co., USA) and 0.01 M ammonium carbonate solution. All solvents were degassed before use.

The compounds investigated were prepared in our laboratories.

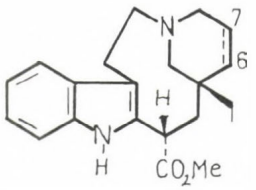
RESULTS AND DISCUSSION

Determination of major and minor alkaloids in Amsonia tabernaemontana. Isolation of the alkaloids from different parts of Amsonia tabrnaemontana needed a number of extraction and chromatographic steps in order to purify the alkaloids from coloured substances and degradation products. The structures of the indole alkaloids isolated from the plant are summarised in Fig. 1.

For the determination of the alkaloid content and the identification of the alkaloids reversed-phase chromatography with octadecylsilica as the stationary phase and acetonitrile-aqueous ammonium carbonate as the eluent can be applied. It seemed a rapid and simple method because, after the exhaustive extraction of seeds with diethyl ether, the coloured extract was purified on WATERS SEP-PAK and directly injected. Fig. 2. shows the chromatogram of tabersonine in Amsonia tabernaemontana seed extract.



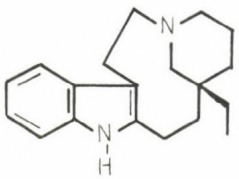
1. (-)-Tabersonine (Δ6,7)



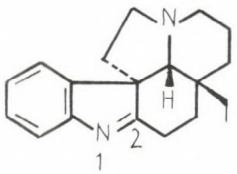
3. (-)-Denydrovincadine (Δ6,7)

2. (-)-Vincadifformine =(6,7 saturated)

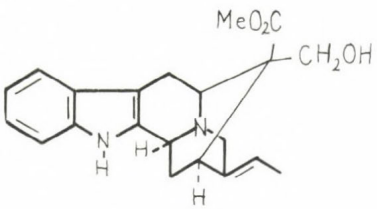
4. (-)-Vincadine (6,7 satd.)



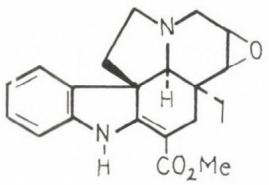
5. (-)-Quebrachamine



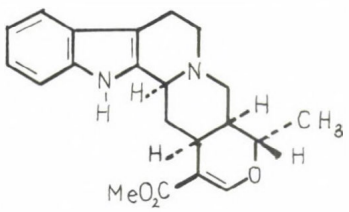
6. (+)-1,2-Dehydroaspidospermidine



7. (+)-Akuammidine



8. (-)-Lochnericine



9. (-)-Tetrahydroalstonine

Fig. 1. Structures of some indole alkaloids isolated from Amsonia tabernaemontana

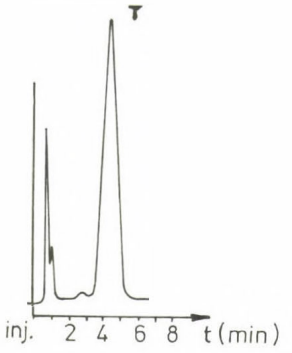


Fig. 2. Determination of tabersonine in Amsonia tabernaemontana seeds. Column: Bondapak C_{18}/Corasil (610 x 2 mm I.D.); eluent: 40 : 60 acetonitrile-aqueous 0.01 M ammonium carbonate; flow rate: 1.5 ml/min; detection at 313 nm

The results of the quantitative determinations were in correlation in the case of chromatographic and preparative analysis.

This method can also be used for the separation of minor alkaloids. Figs. 3 and 4. show the separation of minor alkaloids isolated from seeds and leaves.

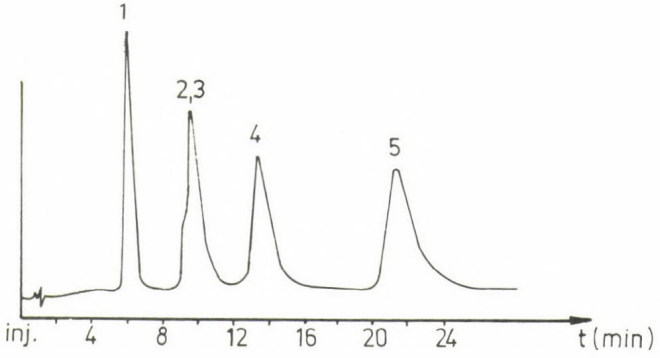


Fig. 3. Separation of alkaloids from Amsonia tabernaemontana seeds. Column: Polygosil C_{18} (250 x 4.5 mm); eluent: 50 : 50 acetonitrile-aqueous 0.01 M ammonium carbonate; flow rate: 3 ml/min; detection: 280 nm. Compounds: 1 = tetrahydroalstonine; 2 = 1,2-dehydroaspidospermidine; 3 = tabersonine; 4 = vincadifformine (internal standard); 5 = quebrachamine

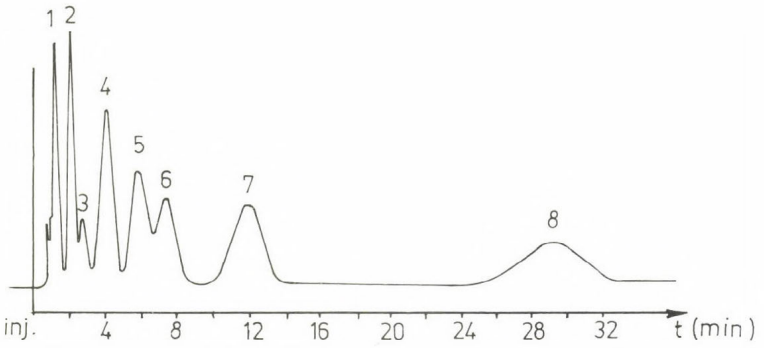


Fig. 4. Separation of alkaloids from Amsonia tabernaemontana leaves. Column: Bondapack C_{18}/Corasil (610 x 2 mm I.D.); eluent: 40 : 60 acetonitrile-aqueous 0.01 M (ammonium carbonate; flow rate: 1.5 ml/min; detection at 280 nm. Compounds: 1 = akuammidine; 2 = lochnericine; 3 = tetrahydroalstonine; 4 = tabersonine; 5 = vincadifformine; 6 = dehydrovincadine; 7 = vincadine; 8 = quebrachamine

It can be seen in Fig. 3. that 1,2-dehydroaspispermidine is eluted with the virtually same retention time as tabersonine. Detecting the alkaloids simultaneously at 280 nm and 313 nm, the amount of 1,2-dehydroaspidospermidine and tabersonine can be determined, because 1,2-dehydroaspidospermidine has no absorption at 313 nm.

Determination of the optimal conditions of the partial synthesis starting from indole alkaloids

Among the modification possibilities of tabersonine the most important is the partial synthesis of vincamine, starting from 6,7-dihydrotabersonine. In the last few years the importance of vincamine in pharmacy has considerably increased. This synthesis is based on the regioselective oxidation of 6,7-dihydrotabersonine. As can be seen in Fig. 5. the oxidation results in 1,2-dehydro-3-hydroxy-3-carbometoxy-aspidospermidine as the intermediary product, which can be transformed to the mixture of vincamine and epivincamine in the presence of an acid:

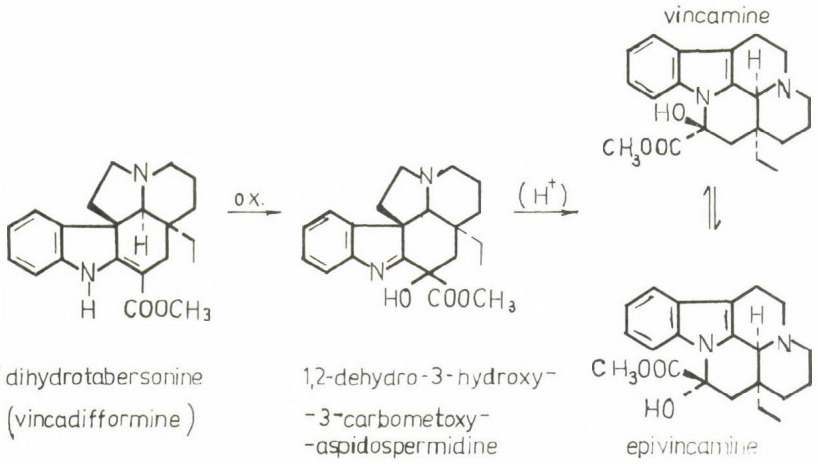


Fig. 5. The regioselective oxidation of 6,7-dihydrotabersonine

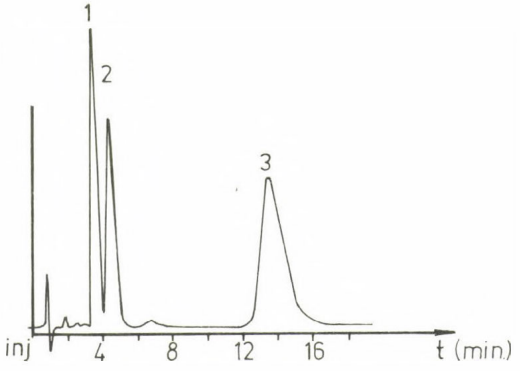


Fig. 6. Chromatogram of standards. Column: Polygosil 60-10 C_{18} (250 x 4.5 mm); eluent: 50 : 50 acetonitrile-aqueous 0.01 M ammonium carbonate; flow rate: 3 ml/min; detection at 280 nm. Compounds: 1 = epivincamine; 2 = vincamine; 3 = vincadifformine

In order to investigate the optimal conditions of this partial synthesis we had to change a number of important factors such as the molar ratio of the acid and the oxidation agent and the temperature. We investigated the presence of possible by-products (6,7-dihydrovincadifformine-N-oxides) during the reaction. Changing the most important factors we

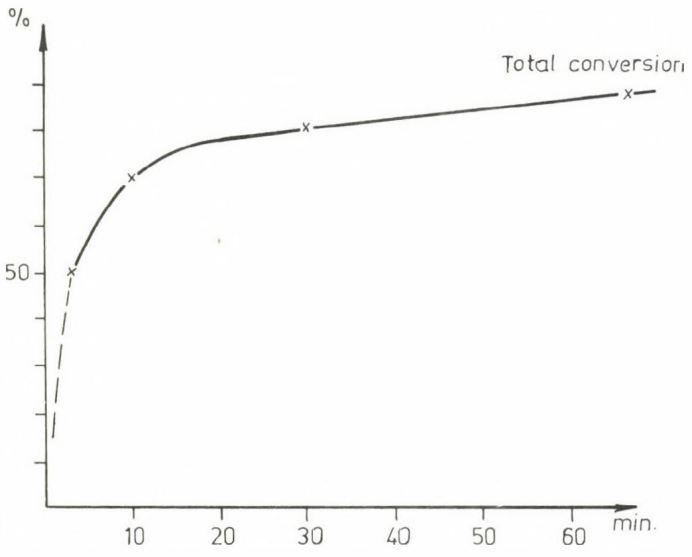


Fig. 7. Dependence of conversion on reaction time

analysed the alkaloids in the reaction mixtures with the help of HPLC and found that the method developed earlier can also be applied here. This is illustrated by Fig. 6.

The results of liquid chromatography made us possible to follow the changing of the conversation with time (Fig. 7.).

Very important is the molar ratio of the hydrochloric acid and vincadifformine (6,7-dihydrotabersonine) in the reaction mixture. The dependence of the conversion on this molar ratio can be seen in Fig. 8.

Investigating the epimer ratio, the chromatograms showed that this ratio changed in time and that it also depends on the molar ratio hydrochloric acid/vincadifformine (Fig. 9.)

As shown the reversed-phase chromatographic investigations gave us some important information in connection with the conditions of the partial synthesis.

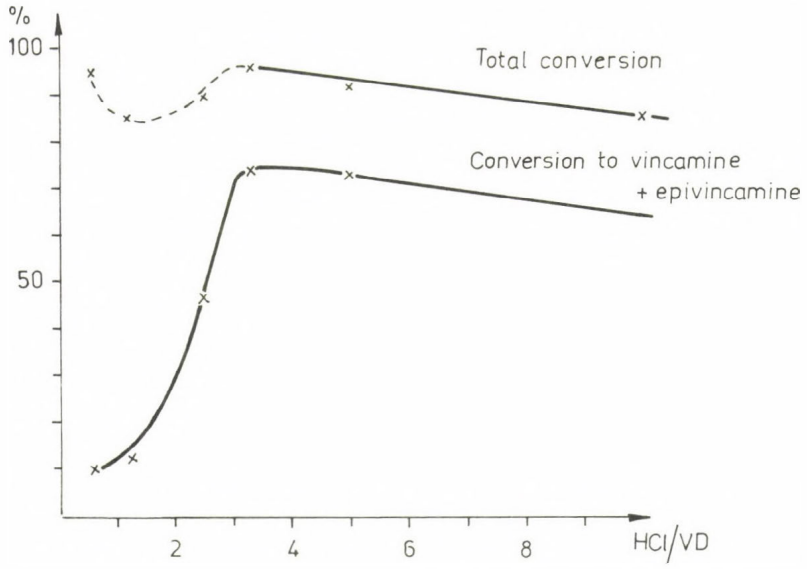


Fig. 8. Dependence of conversion on the molar ratio acid/vincadifformine

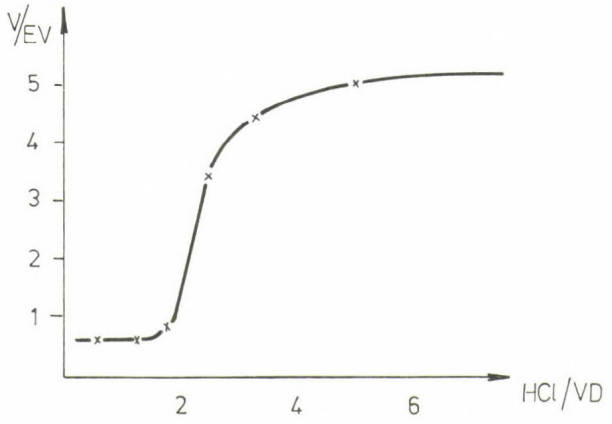


Fig. 9. Dependence of the vincamine-epivincamine ratio on the ratio of hydrochloric acid-vincadifformine

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COMPARISON OF GLYCOLIPID SEPARATIONS BY DIFFERENT LIQUID CHROMATOGRAPHIC METHODS

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INTRODUCTION

Glycosphingolipids, in addition to glycoproteins, are an important group of membrane-associated complex carbohydrates (1, 2).

In recent years the biological functions of glycolipids (3, 4) have attracted much attention making rapid and reproducible isolation and analytical determination of glycolipids necessary for the investigations. Several chromatographic such as e.g., HPTLC (5, 6, 7), HPLC (8, 9, 10) etc. methods have been published for the separation of derivatized or non-derivatized glycolipid samples, but the isolation and detection of pure non-derivatized glycolipids has to be in focus because of their further use for biochemical, immunological etc. experiments. The function of glycolipids (11, 12) has been increasingly examined, parallel with the methodological developments of identification (13, 14) during the last few years.

Our objective was the development of faster and simpler methods than those commonly used for the separation and analysis of blood element glycolipids.

We had to solve the problem of separation for various purposes, namely analytical separation of minute samples with sufficient reliability for possible diagnostic use, and also the semipreparative separation of glycolipids for later biochemical, immunological, serological use.

The two methods described in our present paper, are complementary. HPLC is more suitable for semipreparative separa-

tions, as recovery is easier then from TLC plates, in addition to the the higher purity of the fractionated components, and the possibility for scale-up.

The OPLC technique and an instrument were developed in Hungary (15) and applied to the analysis of various compound classes (16). The application field of OPLC was extended by us to lipid analysis (17). OPLC in contrast to HPLC is useful both for interclass and intraclass separation of minute lipid samples with detection by the suitable staining reaction, permitting several orders of magnitude lower detection limits than refractive index (RI) detection in HPLC. A further advantage of the OPLC method is that the neutral and/or acidic fraction of the lipid extract can be spotted on the plate, developed once for lipid class separation, then the selected class separated in an additional development step, which can be the second step of a gradient or a separate run with the plate rotated at 90 degrees.

We have developed an HPLC method for the semipreparative class fractionation of lipids in non-derivatized form with coupled on-line UV and RI detection (18). For analytical separations of the individual glycolipids we employed OPLC with a modified instrument described previously (17).

EXPERIMENTAL

Reagents and Materials

All the solvents used were HPLC grade purchased from E. Merck, Darmstadt, FRG ; the water used was prepared according to Gurkin and Ripphahn (19), from water double distilled from glass and sterilized. DEAE Sephadex A-25 was obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden. ODS-GU and SIL-GU cartridge columns were purchased from Supelco Inc., Supelco Park, Bellefonte, PA, USA. Staining reagents were prepared from analytical-grade chemicals, and the HPTLC plates used were purchased from E. Merck, Darmstadt, FRG. Total Lipid Extract (TLE) was prepared from human blood elements. Sulphatides (bovine) and GT1b were purchased from Supelco Inc., Supelco

Park, Bellefonte, PA, USA. Other ganglioside standards (GM3, GM2, GM1, GD1a, GD1b) were the kind gift of Professor Shimon Gatt, Hadassah Medical School, Dept. of Biochemistry, Jerusalem, Israel.

Apparatus

Centrifuge: Janetzki K 70 MLW, Leipzig, GDR.

Incubator: LP 507/1 Labor MIM, Esztergom, Hungary.

HPLC system: HP 1084B with HP 79875 variable wavelength UV detector, Hewlett Packard, Palo Alto, CA, USA. Beckman 112 solvent delivery module, Altex 156 differential refractometer, Beckman Instruments Inc., Berkeley, CA, USA.

Fraction collector: LKB Minirac 1700, LKB Produkter AB, Bromma, Sweden.

Sample applicators: special 1 microliter syringe, MTA KKKI Budapest, Hungary and a Camag Nanoapplicator, Camag, Muttenz, Switzerland.

OPLC system: Chrompres 10 Overpressured Layer Chromatograph, Labor MIM, Esztergom, Hungary. Instead of its eluent pump, two HPLC pumps (Beckman 112 SDM, Beckman Instruments Inc., Berkeley, CA, USA.), or one pump and an injector valve (Altex 210 with a 3 ml loop, Beckman Instr. Inc., Berkeley, CA., USA) were used. The block diagrams of the systems were given elsewhere (17a, b).

Spectrophotometric densitometer: Opton KM 3, Opton Feintechnik GmbH., Oberkochen, FRG.

Data system: HP 3354 LAS, HP, Avondale, PA, USA

METHODS

Sample preparation

A model standard glycolipid sample was prepared from bovine sulphatides and pure individual gangliosides (GM3, GM2, GD1a, GD1b, GT1b). The Svennerholm nomenclature for gangliosides (20) is used throughout.

Total Lipid Extract (TLE) preparation: lipid extraction was carried out similarly to the method published previously (21, 22). The TLE was extracted from the lyophilized cells or plasma at room temperature with mild sonication in chloroform: methanol (C:M) mixtures in the ratios of 2:1, 1:1, 1:2. All the supernatants were collected together and dried in vacuo. The dried TLE was dissolved in C : M : W = 30 : 60 : 8 (W=water) mixture (100 ml) and the solution used for the isolation of the lipid classes described below.

Isolation of Acidic and Neutral Lipid Fractions (AcLF and NLF): the separation was performed on a DEAE Sephadex A-25 column (12 mm i.d. x 70 mm) in the acetate form. The solution of TLE (100 ml) was applied to the column, washed with 100 ml of the same solvent followed by 50 ml methanol. The 250 ml solution issuing from the column was used after solvent evaporation (water traces were removed with benzene), for the fractionation of neutral lipids dissolved in chloroform. Acidic lipids were eluted with 50 ml of 0.25 M methanolic sodium acetate. This portion of the effluent was dried in vacuo and incubated in 15 ml of 0.1 M methanolic potassium hydroxide at 37^{o}C for two hours to destroy the alkali labile phospholipids (PL). We have developed a two-step chromatographic method for the recovery and isolation of acidic glycolipids. After the incubation the sample was dried in vacuo and the residue dissolved in cold HPLC grade water, neutralized very carefully with HCl (0.5 M) to pH=4.5. The salt concentration was finally adjusted to 0.1 M by adding water. This sample solution was passed through an ODS-GU cartridge at a flow rate of 1.5 ml/min, the acidic glycolipids being collected on the column. After washing the cartridge with 50 ml of water, the glycolipids were eluted by 30 ml of C : M = 1 : 2 mixture.

Class fractionation of NLF

HPLC method: the neutral lipid portion derived from 2-8 x 10^{10} lymphocyte cells was taken up in 3 ml of dry chloroform and 5-30 µl aliquots were injected. We employed the HP 1084 B LC equipped with UV and RI detectors coupled in series,

with a fraction collector after the last detector. Detection was at 254 nm, reference wavelength 600 nm, attenuation 0.9 AUFS for the UV and 1 V FS for the RI detector. The elution profile selected was: flow 1 ml/min, solvent A was chloroform, B methanol. The gradient started with 1 minute isocratic 100% A, followed by an increase to 100% B in 9 minutes, then a linear decrease to 100% A in 5 minutes; the run ended after further 5 minutes by 100% A. The gradient gave optimal results for class separation and was conducted at ambient temperature. In addition to on-line detection the fractions collected during the run were monitored by parallel TLC runs on silica plates. The fractions containing the same neutral lipid subclasses were united.

OPLC method: sample application was 200 nl/spot with the Camag Nanoapplicator. Usually 12 samples were applied to a plate, 30 mm from the lower edge and at least 20 mm from the perpendicular edges of the plate. Development conditions were: isocratic elution with chloroform, flow rate 0.15 ml/min, start pressure 30 bar, pillow pressure 12 bar, development distance 17 cm. Staining was by immersion into orcinol-sulphuric acid reagent and heating at 100°C for 2 minutes.

Class fractionation of AcLF

HPLC method: the AcLF eluted from the DEAE Sephadex column was dried in vacuo and taken up in 2 ml of C : M = 85 : 15 mixture and applied to a Brownlee Labs SIL-GU column. Sulphatides (S) were first eluted with 15 ml of the same solvent. Gangliosides were eluted with C : M = 2 : 3 mixture. The purity of the ganglioside (G) class separated was checked with HPTLC runs.

OPLC method: the mode of sample application (number, quantity and position) was the same as for the NLF. An indicator solution was applied to the first and last sample spots to help us control the run. Development conditions were: isocratic elution with C : M = 7 : 3 mixture, flowrate 0.1 ml/min, start pressure 20 bars, pillow pressure 14 bars, development distance 7 cm. Staining was by spraying with resorcinol-hydrochloric

acid reagent and heating at 100°C for 15 minutes in HCl atmosphere.

Intraclass fractionation of glycolipids

Development of an HPLC method for the further fractionation of individual glycolipid classes with on-line detection is in progress. For this purpose RI detection used in class separation is not sufficient because of its poor sensitivity. On the other hand UV detection with its greater sensitivity is suitable only for derivatized glycolipids. We are trying other on-line detection techniques giving us sufficient sensitivity for non-derivatized glycolipid samples.

OPLC separations on glycolipid classes can be performed directly from the NLF or AcLF with step-gradient development (17a, b). The first segment of the step-gradient is the isocratic development for the class separation of each fraction as detailed above. Development conditions for the second segment of the step-gradient were:

Glycolipid class	neutral	acidic
Eluent	C:M:W = 60:25:4	C:M:KCl/W = 55:36:9
Flow (ml/min)	0.15	0.10
Start pressure (bar)	30	20
Pillow pressure (bar)	12	14
Development distance (cm)	13	overrun

(KCl/W is 0.25% KCl in water)

The step gradient run means the sharp changeover from isocratic eluent No. 1. to No. 2. To achieve ideal dispersion of the eluents a flow program was used. Staining for Neutral Glycolipid (NGL) separation was by the immersion of the plate in orcinol-sulphuric acid reagent and heating at 100°C for 2 minutes, for the ganglioside separation by spraying the plate with resorcinol-hydrochloric acid and heating at 100°C for 15 minutes in HCl atmosphere.

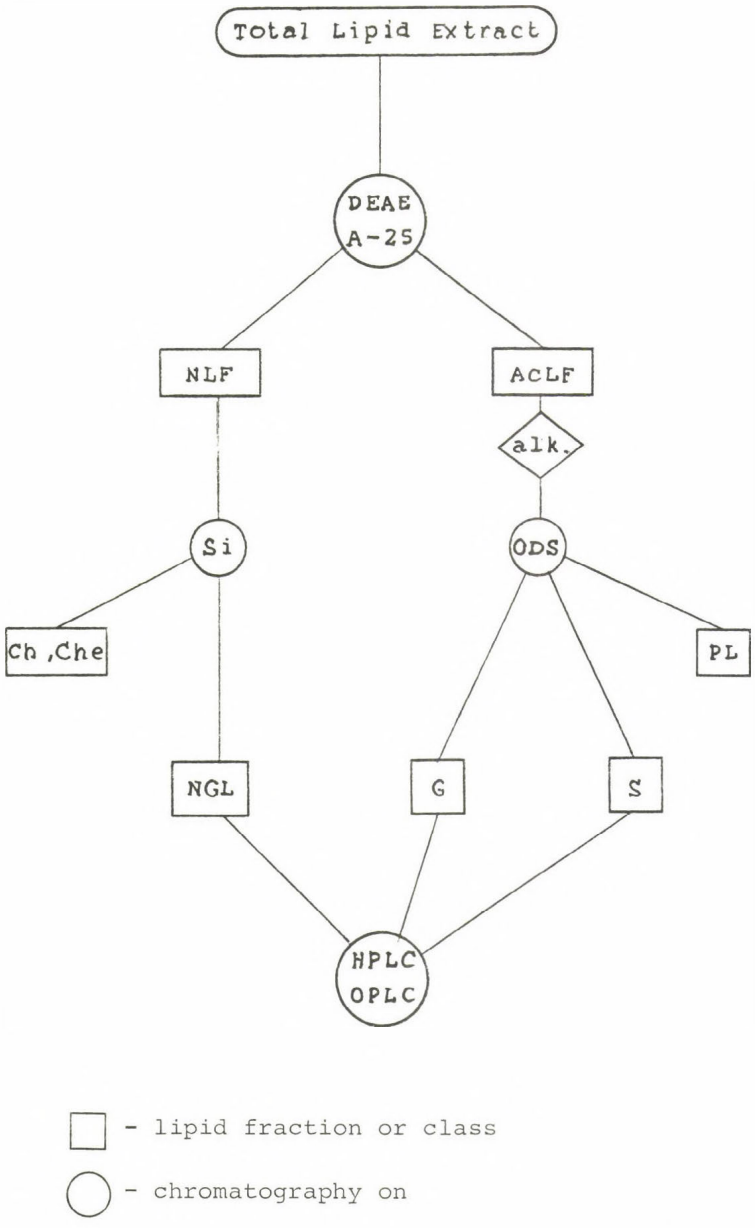

Fig. 1. Flowchart of the lipid separation scheme

Densitometric conditions in remission mode were:

Wavelengths:	560 nm for NGL
	525 nm for G
Slit:	3.5 x 0.1 mm
Scanning speed:	10 mm/min
A/D frequency:	2 Hz

RESULTS AND DISCUSSION

During the last 2-3 years glycosphingolipid research has been extended to more detailed studies into their isolation, structure and biological role. The most recent reviews in the field of biochemical, biological and immunological aspects of gangliosides (23), e.g., their interaction with toxins, the metabolism of ingested gangliosides, their function in synaptic transmission etc. have stressed the importance of ganglioside purity necessary for all investigations.

The trend in analysis and further use of samples from biological origin for various purposes points out the importance of sample pretreatment and preparation (24).

The fully chromatographic method developed in our lab for the separation of TLE into lipid classes, in purity adequate for further fractionation into separate lipid types, has the advantage of speed, the possibility of automation and development into an on-line complete HPLC fractionation.

The first step in our method of TLE fractionation is the known DEAE Sephadex chromatography, as can be seen in the flowchart (Figure 1.), for the isolation of NLF and AcLF.

For the HPLC separation of the two fractions we applied 3 cm long silica and/or octadecyl-silica cartridges. The glycosphingolipid classes isolated in the way outlined above can be further analysed by a suitable column or planar liquid chromatographic method. By processing the lipid samples in this manner we can produce glycolipid classes for at least 6 parallels within two days. The separation was monitored and the

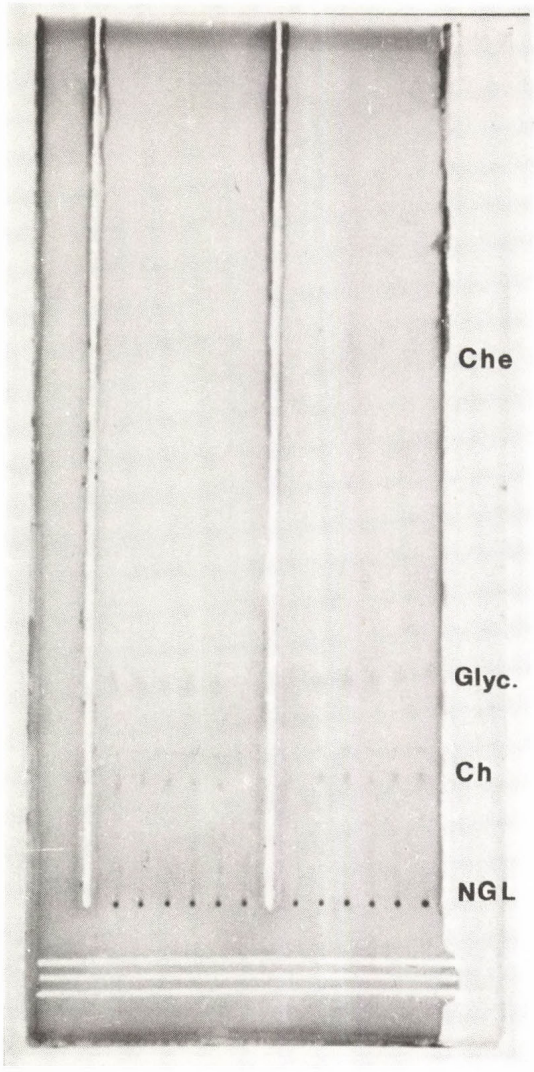

Fig. 2. OPLC separation of the neutral lipid classes. 17 cm elution with chloroform on Merck HPTLC Si 60 plate 10 x 20 cm, stained with orcinol

purity of the neutral glycolipid and ganglioside classes was checked by HPTLC.

The advantage of the OPLC separations developed for analytical purposes is that the NLF or desalinated AcLF can be

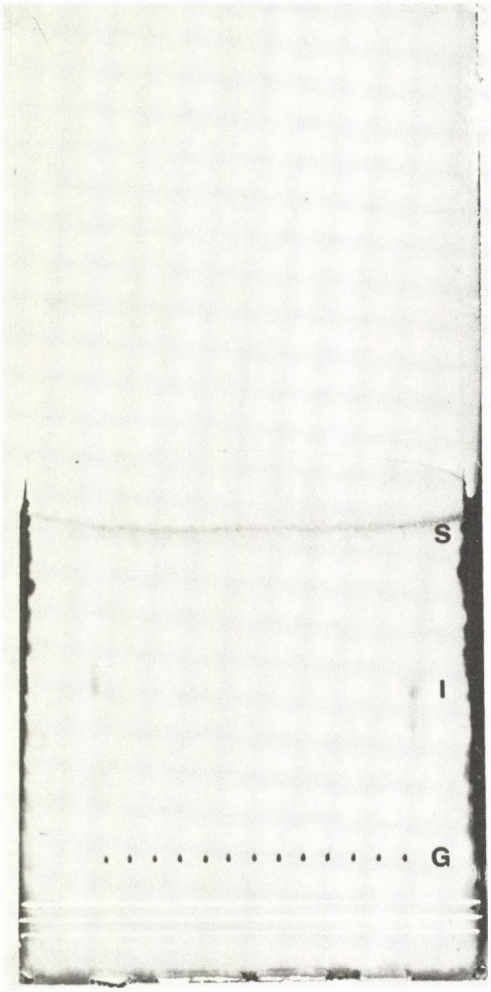

Fig. 3. OPLC separation of acidic glycolipid classes. 7 cm elution with C : M = 7 : 3 on Merck HPTLC Si 60 plate 10 x 20 cm stained with orcinol

directly spotted onto the plate. The class separation of either fraction can be performed by an isocratic run within 15 minutes. If necessary the intraclass separation can be performed, requiring only an additional 15 minutes by step gradient elution, necessitating somewhat more sophisticated apparatus and

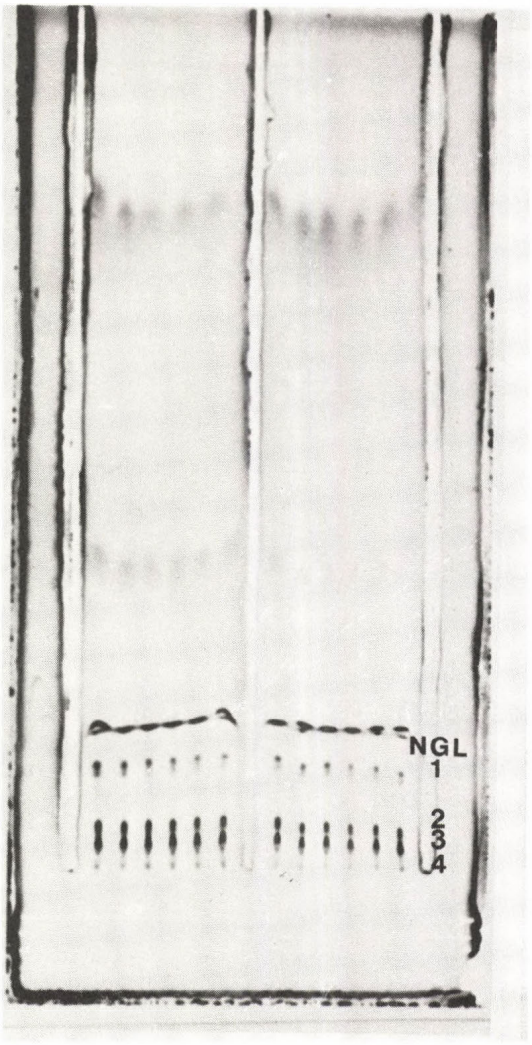

Fig. 4. OPLC separation of neutral glycolipids following class separation. Step gradient elution with C, C : M : W = = 65 : 25 : 4, stained with orcinol

more expertise, for the optimal employment of the flow and time program, (the detailed description of which will be published in the near future). On the following figures we show the class separation of NLF (Figure 2) and AcLF (Figure 3), furthermore the intraclass separation of neutral glycolipids (Figure 4) and

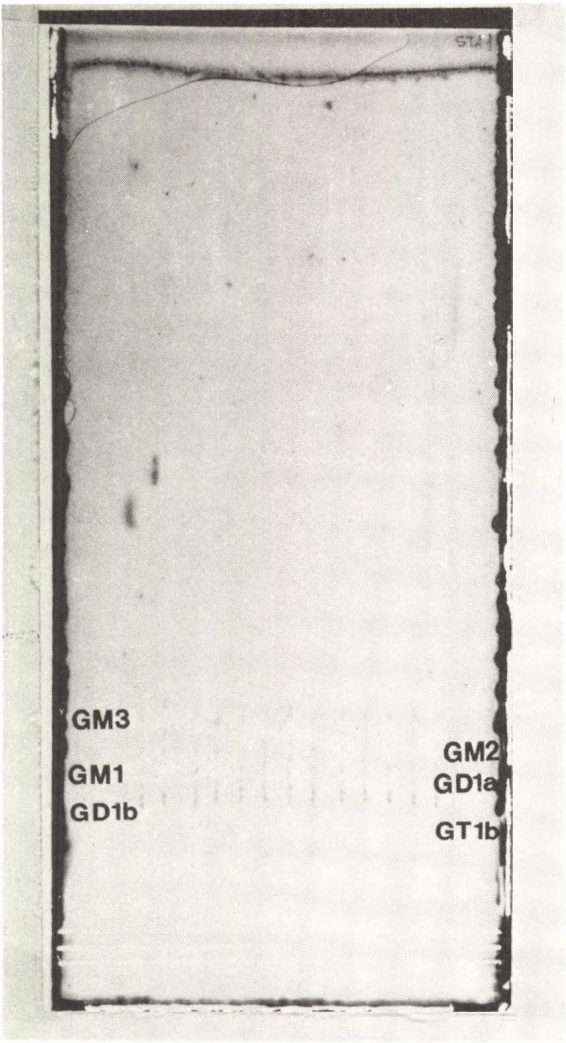

Fig. 5. OPLC separation of gangliosides following class separation. Step gradient elution with C : M = 7 : 3 and C : M : KCl/W = 55 : 36 : 9, stained with resorcinol-HCl

gangliosides (Figure 5). As can be seen from the figures good separations can be achieved for both fractions (NLF, AcLF) for both inter- and intraclass. The chromatograms stained with a suitable reagent can be evaluated by densitometric scanning, collected and processed by an appropriate data system. The data

given in the following tables were all extracted from data collected by our data system. The HPLC data were also transferred to the data system from the LC unit.

Lipid class separations were performed both by HPLC and OPLC. Table 1. contains the comparable data.

Table 1. Reproducibilities of class separation of NLF. Comparison of HPLC and OPLC

Lipid class	HPLC		OPLC	
	t (min)	SD	md (cm)	SD
NGL	6.875	0.130	0.04	0.023
Ch	5.960	0.060	0.36	0.015
Che	0.732	0.016	2.38	0.084

t - average retention time
md - average migration distance
SD - standard deviation
NGL - neutral glycolipid
Ch - cholesterol
Che - cholesterol esters
The data were calculated from 13 parallels.

As can be seen both methods are reasonably reproducible as demonstrated by the standard deviations. Because of other considerations we recommend OPLC for analytical and HPLC for preparative purposes.

Analytical scale intraclass separations of single glycolipid classes (neutral glycolipid and ganglioside) were performed by step gradient OPLC. If two-dimensional development is preferred, the possibility exists, as we showed earlier (17a), but for routine work we used unidimensional step gradient development, which is more productive and economical. The reproducibility of individual neutral glycolipid and ganglioside separations, by two consecutive chromatographic developments in the same direction, are shown below (Tables 2. and 3.).

Table 2. Reproducibility of intraclass separation of neutral glycolipids by OPLC

NGL	md (cm)	SD
NGL1	1.521	0.040
NGL2	1.038	0.048
NGL3	0.725	0.045
NGL4	0.267	0.033

NGL1, 2, 3, 4 are mono-, di-, tri- and tetrahexosyl ceramide.
The data were calculated from 13 parallels.

Table 3. Reproducibility of intraclass separation of gangliosides by OPLC

G	mg	SD
GM3	0.436	0.009
GM2	0.364	0.007
GM1	0.309	0.007
GD1a	0.238	0.010
GD1b	0.193	0.015
GT1b	0.127	0.019

The mg is a specially defined migration factor, analogous with Rf, for overrun OPLC described elsewhere (17b).
The data were calculated from 13 parallels.

As the reproducibilities of the separations into individual species in the neutral glycolipid and ganglioside classes are very good, the OPLC method can be adapted for ganglioside mapping. The standard deviations are good indeed, especially if one considers that they are the cumulative values of two consecutive planar chromatographic separations. Our OPLC method has been introduced for ganglioside pattern determination of leukemic cell lipids.

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A NOVEL, RAPID OPTLC METHOD FOR THE SEPARATION OF SERUM LIPIDS

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SUMMARY

A method capable of separating and measuring lipid fractions of main lipid classes in human sera was developed. The eluents were selected according to Snyder using the PRISMA system. Our system permitted the separation of serum lipids and cholesterol ester subfractions on the same plate within 20 minutes in the following order: phospholipids, free cholesterol, free fatty acids, triglycerides, cholesterol ester subfractions I, II, III, IV by CHROMPRESS 10.

INTRODUCTION

The investigation of serum lipids, mainly cholesterol ester subfractions has clinical importance in differential diagnosis of hepatic diseases and lipid metabolism disorders. In addition hyperlipemia has an outstanding place among the risk factors of cardiovascular diseases. Therefore, the study of lipid metabolism is an important application field of thin-layer chromatography (1, 2).

Until now two problems have been encountered. The TLC techniques were not satisfactory for the separation of neutral lipids and cholesterol ester subfractions in a single chromatogram (3, 4). The solvent systems generally used for classical thin-layer chromatography do not provide a good separation of the cholesterol esters and are not satisfactory for lipid

classification by OPTLC (overpressured thin-layer chromatography) (5). There is an OPTLC method which is suitable for the class separation of the neutral lipids (6), however, it cannot separate simultaneously the cholesterol.

The present method is simple and fast for the separation of main lipids and cholesterol esters.

MATERIALS AND METHODS

Solvents and materials

Reagent-grade chemicals and solvents were used. The eluents were not purified prior to use. Toluene was purchased from Merck (Darmstadt, G.F.R.), carbon tetrachloride was from Reactivul Bucuresti (Bucuresti, Rumania). All other chemicals were obtained from Reanal (Budapest, Hungary).

The following solvent systems were used: System I.: toluene-carbon tetrachloride (1:3, v/v); System II.: plain toluene.

Plates

TLC pre-coated silica gel (Merck No. 5721, 20x20 cm, 250 m) and Nano-Platten SIL-20 UV_{254} (zur Dünnschicht-Chromatographie 10x20 cm, layer: 0.20 mm Kieselgel 60 with fluorescent indicator, Macherey-Nagel Co., D-5160 Düren, G.F.R.) were used. The plates were activated before use in a heating box (at 130 ^{o}C, for 30 min). The OPTLC technique requires the edges of the plates to be sealed by impregnating them with paraffin. For the development three sides of the plate were sealed into the plates perpendicular to the development direction. Standard and sample solutions (5 µl) were applied with a Hamilton syringe (Bonaduz, Switzerland) at 1.5 cm up from the lower edge of the silica gel layer.

Lipid standards

The following reference lipids were obtained from Reanal (Budapest, Hungary); cholesterol, free fatty acids and triglycerides. Solutions were prepared according to the standard procedure (3) resulting in 1 mg/ml concentrations of the reference material in the solutions. The identification of the fatty acid methyl ester peaks was performed using Supelco GC-FAME standards (Bellefonte, PA, USA).

Development procedure and instruments

The CHROMPRESS 10 instrument made by Labor MIM (Budapest, Hungary) was used in our work. The eluent pump was filled with solvent system I (cca. 10 ml) before the development, and the filling head of the pump was taken into solvent system II. The chromatograms were developed at room temperature up to the edge of the plates (cca. 20 min). The inlet pressure was 0.2 MPa at the start, the solvent flow rate was 0.2 ml/min and the overpressure was 2.0 MPa. The instrument was operating at optimum solvent flow (7). After migration the plates were allowed to dry at room temperature. The spots were detected by spraying the plate with the developing solvent (5 g phosphomolybdic acid dissolved in 100 ml ethanol + 1 ml perchloric acid), then heating in an oven at 100 ^{o}C for at least 2 min. The blue spots of the lipids againts the yellov background may be investigated easily.

Selection of the best eluent system

The most satisfactory eluent system was developed with the help of the PRISMA system (8). This is a procedure for mobile phase optimization where the solvents are compared based on their strength and selectivity according to Snyder (9). In our OPTLC study we first selected one solvent from each selectivity group and the best solvents were chosen for further analysis. The optimization of the solvent composition was determined by PRISMA; the solvent composition for OPTLC separations was mod-

elled using UM chambers. The optimal solvent composition was applied for the OPTLC assay in order to obtain a better selection in a short development time. PRISMA is very efficient in the optimization of OPTLC separation.

Serum samples

The blood samples were taken from patients with liver diseases after 12 h fasting. The serum lipids were extracted from 2 ml of serum according to Folch et al. (10). The study material was maintained at -40 ^{o}C before use. The dried extract was dissolved in 1 ml chloroform-methanol (1:1, v/v) and 10 µl of this solution were spotted on the plate at the place marked with S in the figures.

Identification of cholesterol ester subfractions

Cholesterol esters were purified by OPTLC. The separated spots were scrapped and eluted off the gel with 3 ml acetone, then mixed and centrifuged (for 30 min, 3000/min). The supernatant was dried in a nitrogen stream. The fatty acid content of the cholesterol esters was checked by gas chromatography (GC) after derivatization to the methyl esters. The fatty acid methyl esters were determined using a Hewlett-Packard Model, 5840/A gas chromatograph in a comparative system id. = 0.3 mm, standard thickness, (system I.) and on a 25 m x 0.2 mm i.d. fused-silica capillary column, with 0.11 µm film thickness (system II.).

The chromatographic parameters were:

System I.: temperature program from 100 ^{o}C to 175 ^{o}C at 15 ^{o}C/min, and from 175 ^{o}C to 220 ^{o}C at 4 ^{o}C/min. FID temperature: 250 ^{o}C; injector temperature: 280 ^{o}C, carrier gas: N_2 (1.0 bar), direct injection.

System II.: temperature program from 100 ^{o}C to 300 ^{o}C at 8 ^{o}C/min, FID temperature: 300 ^{o}C, injector temperature: 300 ^{o}C, carrier gas: N_2 (0,8 bar), direct injection.

RESULTS AND DISCUSSION

As the laboratory investigations show the lipid disorders are essentially manifested as hypercholesterinemia or hypertriglyceridemia, sometimes both together. In a normal human serum 70-75% of the total cholesterol is esterified. In patients with liver disease the percentage of esterified cholesterol may vary to a considerable extent and is often lower.

The complete chromatographic separation of serum lipids mainly for cholesterol esters has been unsolved, because of the presence of a large number of structurally related components in the serum.

We developed a new and sensitive method for the detection of serum lipids and cholesterol ester subfractions by OPTLC. In order to decrease the developing time and drying time nonvolatile compounds (water, acetic acid) or multiple compounds were avoided. The efficient separation of serum lipids was accomplished by two different but very simple solvent systems. This method has been succesfully applied for the separation of serum lipids. The advantages of the OPTLC migration with the new solvent system are illustrated in Fig. 1 showing the separation obtained with solvent system I. and II. If solvent system I. is used alone (Fig. 2.) the separation between free cholesterol and triglycerides is not satisfactory.

If normal kieselgel plates were used in the CHROMPRESS 10, the cholesterol ester separation was poor: only three fractions could be observed (Fig. 3).

When analyzing serum samples of ill patients, the cholesterol ester spots are larger than in the case of a sample originated from a healthy individual. This is due to the fact that in liver diseases the blood serum contains several cholesterol esters.

We found that the OPTLC system is suitable for the determination of serum lipids and cholesterol ester subfractions; the diffusion of the spots is smaller, and the reproducibility is very good. The complete and well-detected separation of the neutral lipids with this method is suitable for quantitative determinations.

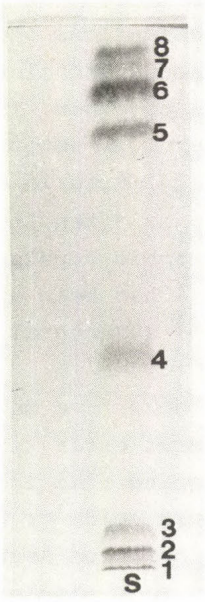


Fig. 1. Thin-layer chromatogram of lipids developed in solvent system I + II. 1 = phospholipids, 2 = cholesterol, 3 = tryglycerides, 4 = free fatty acids, 5, 6, 7, 8 = cholesterol esters (Nanoplatten)

Fig. 2. Thin-layer chromatogram of lipids developed in solvent system I. For abbrevations see the legend of Fig. 1. (Nanoplatten)

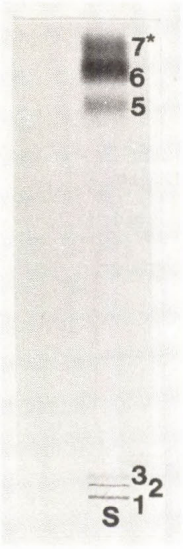


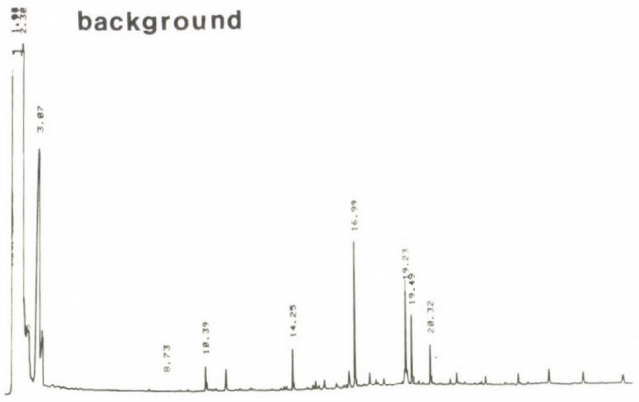
Fig. 3. Thin-layer chromatogram of lipids developed in solvent system I + II. For abbrevations see the legend of Fig. 1. (Merck TLC plate). 7^{+} = cholesterol esters, 7, 8-fractions

GC analysis of the TLC spots showed that the cholesterol esters are separated on the TLC-plates according to the degree of unsaturation of the fatty acids (see Table I, Fig. 4).

Table I. TLC-fraction number and fatty acid composition

TLC-fraction number	Fatty acid
5	18:4, 20:4
6	18:2, 20:2
7	16:1, 18:1
8	16:0.

In conclusion it can be stated that the basis of the correct clinical diagnosis and efficient therapy may be a clarification of hyperlipemic pathomechanism. Early detection of sterol abnormalities is becoming increasingly important. The newly developed method is fast, simple and represents an ef-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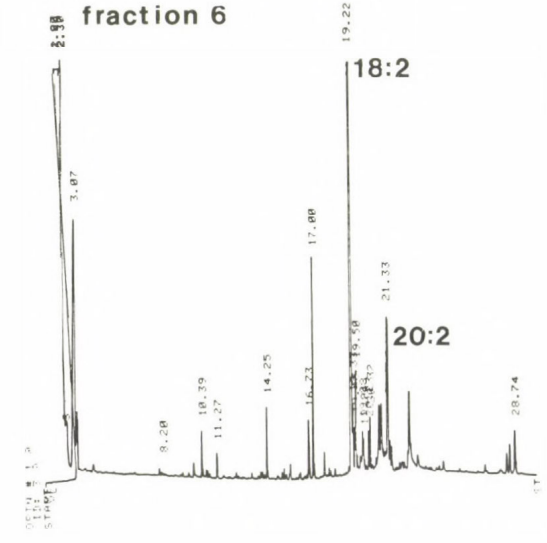
8.73
10.39
14.25
16.99
19.23
19.45
20.32


fraction 6
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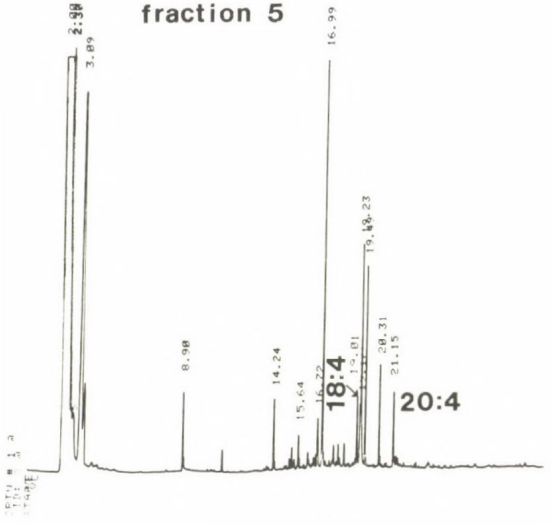
20:2
3.07
8.20
10.39
11.27
14.25
16.23
17.00
19.22
21.33
28.74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20:4
3.09
8.98
14.24
15.64
16.99
21.15

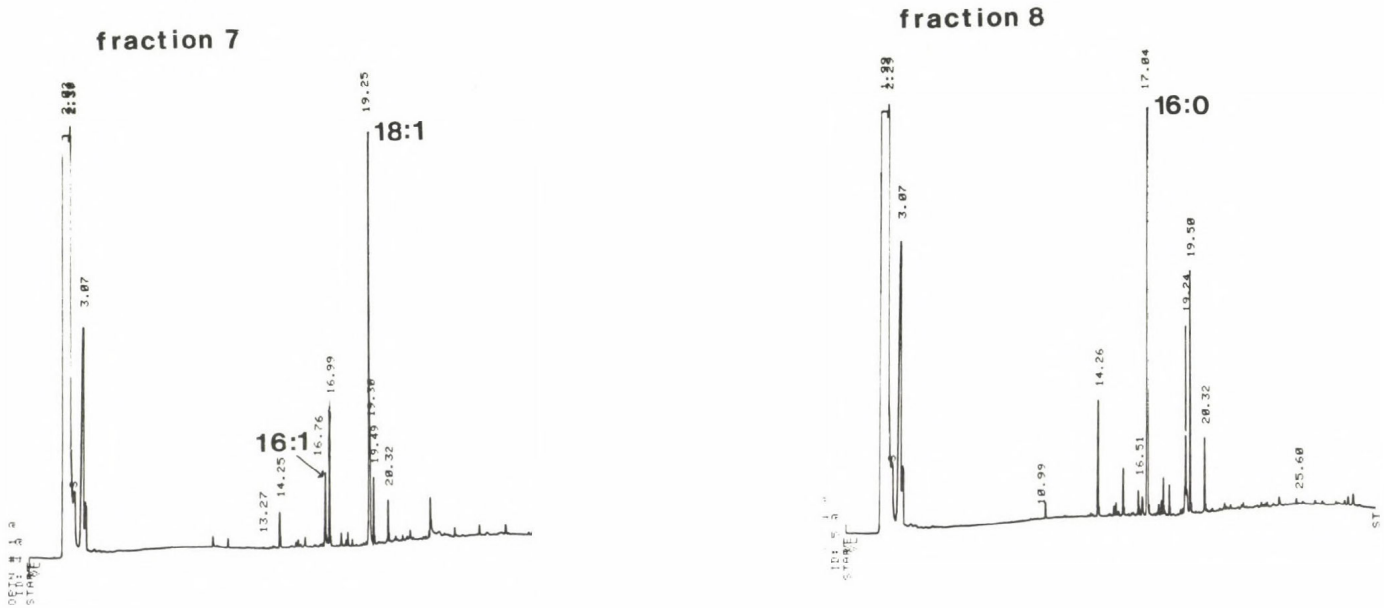

Fig. 4. Gas chromatogram of the fatty acid methyl esters of the cholesterol ester subfractions. System II.

fective way to separate the neutral lipids and cholesterol ester subfractions. It will be useful for studying the clinical significance of these serum components in various diseases.

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RAPID SEPARATION AND QUANTITATION OF SOME GLYCOLYTIC METABOLITES FORMED DURING ENZYMATIC REACTIONS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Fructose-1.6-bisphosphate (FDP) has been converted enzymatically into 3-phosphoglycerate (PG) via glyceraldehyde-3-phosphate (GAP), and these metabolites have been separated by HPLC, utilizing a SynChropack AX 300 anion exchange column with 50-200 mM KH_2PO_4, pH 2.5-4.6, as the mobile phase. The best resolution was reached in a system of 150 mM KH_2PO_4, pH 2.5. The decomposition of metabolites during the chromatographic procedure was excluded by enzymatically testing the fractions. If radioactive FDP as the initial substrate was converted into PG as the end product by coupled enzymatic reaction, the recoveries of metabolites after the precipitation and chromatographic procedures were higher than 95 per cent. The concentration of radioactive PG measured by liquid scintillation agrees with the spectrophotometrically determined concentration of NADH, which is formed in equimolar concentration with PG. These results indicate that the method developed is applicable for the quantitative determination of these glycolytic metabolites during enzymatic reactions.

INTRODUCTION

The formation of a kinetically significant complex between two functionally related enzymes, aldolase and glyceraldehyde-3-phosphate dehydrogenase (GAPD) has already been propounded

/1/. The direct transfer of the intermediate from the active site of one enzyme to that of the other can be performed by the "isotope dilution" technique, i.e. by measuring the specific radioactivity of the final product formed from radioactive substrate and from unlabelled intermediate /2/.

For this purpose the separation and quantitation of metabolites involved in the reactions are required.

FDP, GAP and dihydroxyacetone phosphate (DHAP) have been separated by precipitation with organic solvents /3/, however, this method was developed for preparative application. HPLC has for some years shown its value in the analysis of amino acids and nucleotides, but no general rules have been established that would allow the simultaneous separation of all monosaccharides due to the sheer multiplicity of the structures /4/. Moreover, no effective and convenient analytical methods are available for the detection of carbohydrates although a number of procedures have been proposed. Most of them give rise to derivatives having aromatic and heterocyclic substituents which absorb in the ultraviolet region or fluoresce intensively.

In the present work a rapid and sensitive method has been developed for the separation and quantitative determination of metabolites during the enzymatic reaction.

MATERIALS AND METHODS

FDP, GAP, DHAP, PG, NAD and NADH were Boehringer products. All other chemicals were reagent grade commercial preparations. /U-^{14}C/ FDP (specific radioactivity 11077MBq/mM) was a product of UVVVR (Prague, Czechoslovakia). The enzymes, aldolase /5/, GAPD /6/, and phosphoglycerate kinase (PGK) /7/ were purified from rabbit skeletal muscle. GAPD and aldolase were practically free of TPI contamination. Triosephosphate isomerase (TPI) was purchased from Boehringer.

Concentrations of the enzymes were determined spectrophotometrically by using the absorption coefficient $A_{280}^{0.1\%} = 1$ for GAPD /8/, $A_{280}^{0.1\%} = 0.74$ for aldolase /9/, and $A_{280}^{0.1\%} = 1.32$ for TPI /5/.

The concentrations of FDP, GAP, and DHAP were determined by enzymatic assays in the presence of excess NAD and arsenate /10/. The concentration of PG was checked by coupled reaction catalysed by PGK and GAPD /7/. The changes in absorbancy at 340 nm were followed up to the maximum and the concentrations were calculated by using the molar extinction coefficient of NADH /11/.

Enzymatic assay for consecutive reaction

The consecutive enzymatic reaction was carried out at 20°C in 40 mM TEA buffer, pH 8.5. The assay mixture contained 1.12 mM /U-^{14}C/ FDP (specific radioactivity 5.55 MBq/mM), 6 mM NAD, 4 mM arsenate, 30 nM aldolase and 50 nM GAPD. The reaction was started by adding aldolase and was followed by monitoring NADH formation at 340 nm. 0.2 ml of bovine serum albumin (BSA) (10 mg/ml), then 0.2 ml of perchloric acid (PCA) (70%) were added to 2.5 ml of sample to stop the reaction. The samples were stored for 20 min at 4°C, then centrifuged for 15 min at 6000xg. 20 µl of the supernatant was analysed by HPLC.

Equipment and procedures for HPLC

Liquopump OE 312 (Labor MIM, Budapest, Hungary) high-pressure liquid chromatograph was used in these studies. Samples were introduced into the column through a 20 µl Rheodyne 7010 injector (Cotati, Cal., USA). The Labor MIM OE 308 ultraviolet spectrophotometer was equipped with a 10 µl flow cell (detection wavelength: 230 nm). The column used for the analysis was a SynChropack AX300 anion exchanger 250x4.1 mm I.D. (SynChrom Inc., Linden, Ind., USA). The apparatus was operated at room temperature, at about 7MPa (70 bar) inlet pressure, which gave a flow rate of 0.86 ml/min. Isocratic elution was used with a mobile phase of 50-200 mM KH_2PO_4; the values of pH (2.5-4.6) were adjusted by addition of phosphoric acid.

Detection of radioactivity

The radioactivity of the samples was determined in a LKB-Wallace 1211 RackBeta liquid scintillation counter by using a scintillation solution made by dissolving 100 g of 2,5-diphenyl-oxazole and 1.25 g of 1,4-bis(4-methyl-5-phenyl-2-oxazole)-benzene in 1 L of toluene (3% v/v, in a 2:1 mixture of toluene and triton X-100. The ratio of the volumes of scintillation cocktail and sample was 10:1. The efficiency of the counting was determined by using a ^{14}C external standard.

RESULTS AND DISCUSSION

The retention times of the four glycolytic metabolites are shown in Table 1, for an isocratic elution system at different pHs and ionic strengths. Since the metabolites studied do not have appreciable UV absorption, detection limits for screening experiments can only be improved by increasing their concentration.

The concentration of the injected solutions was 20-100 mM. Detection wavelength: 230 nm.

It can be seen from Table 1 that the best resolution can be reached at pH 2.5 and 4.6 in 150 mM and 200 mM KH_2PO_4, respectively. The two triosephosphates, GAP and DHAP, nearly co-elute in these systems, however, their separation has not been aimed. It should be noted that the position of peaks, especially in the case of FDP having relatively high retention time, can be slightly shifted due to the large amounts of metabolites used in these experiments.

The order of separation of FDP and PG is reversed by changing the pH of the eluent. This phenomenon can be attributed to the circumstance that both phosphate groups of FDP are completely deprotonated at both pH 2.5 and 4.6 /12/, whereas the carboxylic group of PG is in the protonated form at pH 2.5 and it is practically dissociated at pH 4.6 /12/. FDP probably binds to the anion exchange resin at two points. Nevertheless, elution of these compounds is influenced by the partition of

Table 1. Retention times of hexose- and triosephosphates at different pHs and ionic strengths

pH	KH_2PO_4 (mM)	Retention times (min)			
		GAP	DHAP	PG	FDP
2.5	50	8.2	9.0		>40
	100	5.4	5.8	14.2	≈40
	150	4.6	4.8	11.0	17.0
	200	4.2	4.4	8.0	12.1
2.6	100	5.4	5.8	16.5	≈40
2.8	100			20.5	≈40
3.2	50	9.3	9.6		>40
	120			23.5	21.2
3.5	50	9.6	9.8		>40
4.6	200	4.3	4.4	16.0	11.2

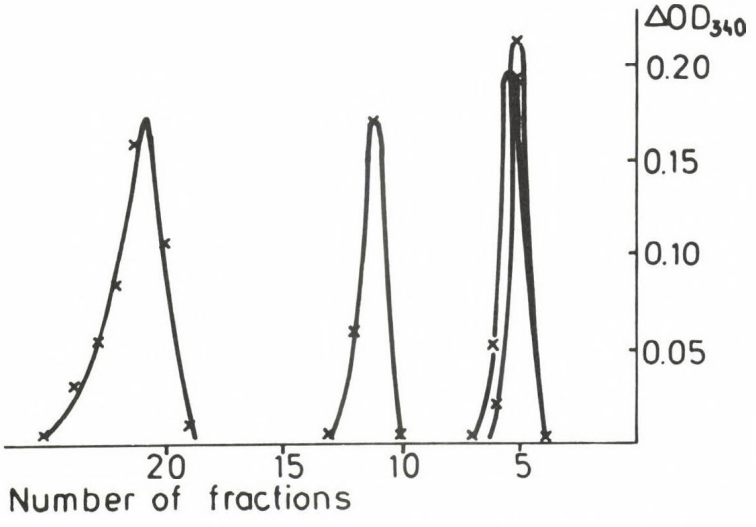

Fig. 1. Separation of hexose- and triosephosphates by HPLC. Mobile phase: 150 mM KH_2PO_4, pH 2.5. The concentration of injected samples was 5 mM. Metabolite concentration of fractions was determined by enzymatic assay. For all other conditions see Materials and Methods.
1 = GAP, 2 = DHAP, 3 = PG, 4 = FDP

these metabolites in the resin and by the relative affinities of the sample ions and eluent ions for the active sites of resin. The good resolution is particularly advantageous when rather large amounts of one of the metabolites (FDP) is present and the others occur in considerably lower concentrations (cf. condition of enzymatic reaction).

Not only the retention times have been used to identify the eluted metabolites, but after separation by HPLC the fractions were also enzymatically tested. (Cf. Materials and Methods). Fig. 1 shows the elution profile of the metabolites studied. The asymmetric shape of the peaks is probably caused by the high concentrations which may exhaust much of the separator column capacity so that all of the ions cannot compete effectively for the exchange capacity.

The injected amount of metabolites (10^{-7}-10^{-6} moles) is practically fully recovered (>95%), thus any decomposition of either FDP or PG can be excluded during the chromatographic procedure.

In order to determine the concentration of the end product, PG*, at a predetermined time, the consecutive reaction was stopped with perchloric acid in the presence of bovine serum albumin (BSA) to ensure the complete precipitation of the enzymes /13/. In control experiments, when the solution did not contain enzymes we found that the concentrations of metabolites did not decrease during this procedure due to either the effect of perchloric acid or aspecific adsorption on the denatured protein. The recoveries of FDP and PG were higher than 95 per cent.

If radioactive FDP was applied as the initial substrate in the enzymatic reaction the sensitivity of detection was enormously increased (the loaded amount of FDP was 10^{-13} moles). The metabolites can be identified easily on the basis of their retention times. The samples were assayed by scintillation counting after the precipitation procedure and chromatographic separation.

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*The end product of the enzymatic reaction catalysed by GAPD in the presence of arsenate instead of inorganic phosphate is PG.

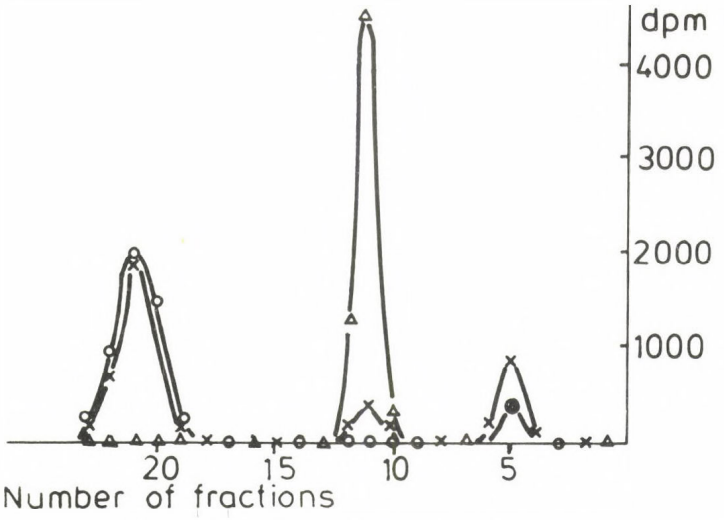

Fig. 2. Elution profiles of metabolites produced by coupled enzymatic reaction.
Mobile phase: 150 mM KH_2PO_4, pH 2.5. The total volume of fractions (0.86 ml) was used for scintillation counting. For all other conditions see Materials and Methods. o——o : ($U^{-14}C$)-FDP in the absence of enzymes. x——x : $U^{-14}C$)-FDP in the presence of aldolase and GAPD; the reaction was quenched at 5 min. Δ——Δ : ($U^{-14}C$)-FDP in the presence of aldolase, GAPD and TPI; after completion of the reaction.
1 = GAP + DHAP + radioactive impurity; 2 = PG, 3 = FDP

Table 2. Concentrations of metabolites of the coupled enzymatic reaction at different stages

Enzymes				Metabolites			
Aldolase (M)	GAPD (M)	TPI (M)	time (min)	FDP[a] (M)	DHAP+GAP[a] (M)	PG[a] (M)	NADH[b] (M)
-	-		0	1.12×10^{-3}	0	0	0
3×10^{-8}	5×10^{-8}	-	5	8.39×10^{-4}	2.98×10^{-4}	2.62×10^{-4}	2.70×10^{-4}
3×10^{-8}	5×10^{-8}	7×10^{-7}	30	0	0	2.24×10^{-3}	2.22×10^{-3}

The initial concentration of radioactive FDP was 1.12×10^{-3} M in each case. Methods of determination: a - scintillation counting, b - spectrophotometry.

Fig. 2 shows the elution profiles of metabolites produced by the coupled reaction.

The enzymes were incubated with their substrates, during which NADH formation was followed spectrophotometrically and aliquots of the incubation mixture were removed at appropriate times and analysed by HPLC after precipitation of the proteins. At the end of the reaction run it the presence of triose-phosphate isomerase to convert DHAP into PG via GAP formation the peak at the position of FDP disappeared and the predominant part of radioactivity appeared at the position of PG. About 10 per cent of radioactivity was detected at the position of GAP+ DHAP, which could not be converted enzymatically into PG. (This inactive contaminant of radioactive FDP was taken into account in the calculations.) If the reaction was quenched during the enzymatic process, the converted amount of radioactive FDP could be recovered as the sum of radioactivities of PG and DHAP + GAP.

The results of these experiments are shown in Table 2.

From this table it can be also seen that the concentrations of NADH measured spectrophotometrically are in a good agreement with the concentrations of PG determined by radioactivity after precipitation and chromatographic procedures. This finding corresponds to the expectation that NADH and PG are formed in equimolar amounts which indicates the reliability of the suggested method.

These results show that the analytical method developed here makes it possible to apply the method of isotope dilution to the investigation of the mechanism of interaction between aldolase and GAPD. Such investigations are in progress.

ACKNOWLEDGEMENT

The column and the injection valve were kindly provided by Chromatronix Inc., California, USA.

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A NEW METHOD FOR THE EXTRACTION AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF VICINE AND CONVICINE IN FABABEANS

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SUMMARY

A new rapid extraction method and a simple high-performance liquid chromatographic assay were developed for the quantitative determination of vicine and convicine, two pyrimidine-glucosides present in fababeans. The method provides a possibility for monitoring the content of the two toxic glucosides in fababean samples.

INTRODUCTION

Fababeans (Vicia faba L.) have a relatively high protein content (20 - 30%) of good quality. However, their use either for human consumption or for animal feeding is limited by several toxic compounds present in the beans. Among them vicine and convicine induce haemolytic disease in individuals deficient in red blood cell glucose-6-phosphate dehydrogenase /1/. Vicine, when consumed by laying hens, causes a marked increase in the level of lipids and lipidperoxides /2/, moreover, the egg-weight production is decreased /3/. As the nutritive properties of fababeans are considerably influenced by the presence of these toxic glucosides a simple and rapid method for the determination of vicine and convicine content is of great importance.

Previously described methods /2-9/ for the extraction and determination of vicine and convicine in fababeans are rather complicated and time-consuming.

In this paper a rapid and simple method is described for the extraction and high-performance liquid-chromatographic (HPLC) analysis of vicine and convicine in fababeans.

MATERIALS AND METHODS

Vicine and convicine reference samples were isolated and purified from dehulled Vicia faba L. var. major by the procedure of Brown and Roberts /8/.

All chemicals used in this investigation were reagent grade and were purchased from Reanal (Budapest, Hungary), if not indicated otherwise.

Extraction of vicine and convicine

Dehulled beans were milled in a pin mill to pass a sieve of 12 mesh. Then 25 ml of a 1:1 (v/v) methanol/water mixture were added to 1 g meal and the suspension was subjected to two successive microwave irradiations for 30 seconds each time. The microwave irradiation was performed in a Toshiba ER (Japan) 638 EDT type microwave oven (U = 220 Volt, P = 1140 Watt, f_o = 2450 MHz). The suspension was then centrifuged at 11 000 x g for 10 minutes. To 1.9 ml of the supernatant 0.1 ml of internal standard stock solution made of 0.1 g sulfaguanidine (Alkaloida Pharmaceutical Works, Tiszavasvár, Hungary) in 100 ml of water was added and mixed thoroughly. 20 µl of the sample solution was subjected to HPLC analysis. The vicine and convicine content and the standard deviation were calculated from six independently prepared samples and each sample was analysed twice by HPLC.

Apparatus

The HPLC system consisted of a Liquopump Model 312 pump (Labor MIM, Budapest, Hungary), a Rheodyne Model 7010 sample injection valve with a 20 µl loop (Cotati, California, USA), a Dimesil LC-18 250 x 4.6 mm column (Chromatronix Inc., Mountain

View, California, USA), an ISCO Model 226 absorbance monitor with 280 nm source screen ISCO Inc. Lincoln, Nebraska, USA) and an ENDIM Model 621.01 (Berlin, GDR) recorder. The integration procedures were carried out on an Apple II microcomputer (Apple Microcomputer Inc., Cupertino, California, USA).

Separating conditions

The analysis of the vicine and convicine content in the bean samples was carried out in a reversed-phase HPLC system using a modified method of Lattanzio et. al. /9/. HPLC grade water (without any organic modifier) was used as the eluent. The flow rate was 1.00 ml/min causing a pressure of 35 $\pm$1bar in the column. The sulfaguanidine solution described above was used as the internal standard. 20 µl of the sample solutions were injected onto the column. The UV absorbance of vicine and convicine at 280 nm was used for detection. The retention time of vicine and convicine was 6.1 and 7.2 minutes, respectively.

RESULTS AND DISCUSSIONS

The reversed-phase chromatographic method using water as the eluent without any organic modifier was found to be suitable for the separation of vicine and convicine (Fig. 1a.). For the extraction of vicine and convicine from fababeans acidic /2, 4, 5/ or alkaline /6/ procedures were recommended. According to our experience, when the pure reference vicine and convicine were kept in acidic (pH = 1.5 with 1 N HCl) or in alkaline (pH = 9 with 0.1 N NaOH) medium for 20 minutes, a considerable decrease of the vicine and convicine content was observed and breakdown products were also detected (Fig. 1b, 1c). Therefore the alcohol-water extraction suggested by Lattanzio et al. /9/ and Jamalian /10/ was chosen.

In order to eliminate the lengthy Soxhlet-extraction step /9/, microwave irradiation of the methanol/water suspension of the milled beans was introduced. The suspension started to boil after 30 seconds of microwave irradiation, then irradiation was

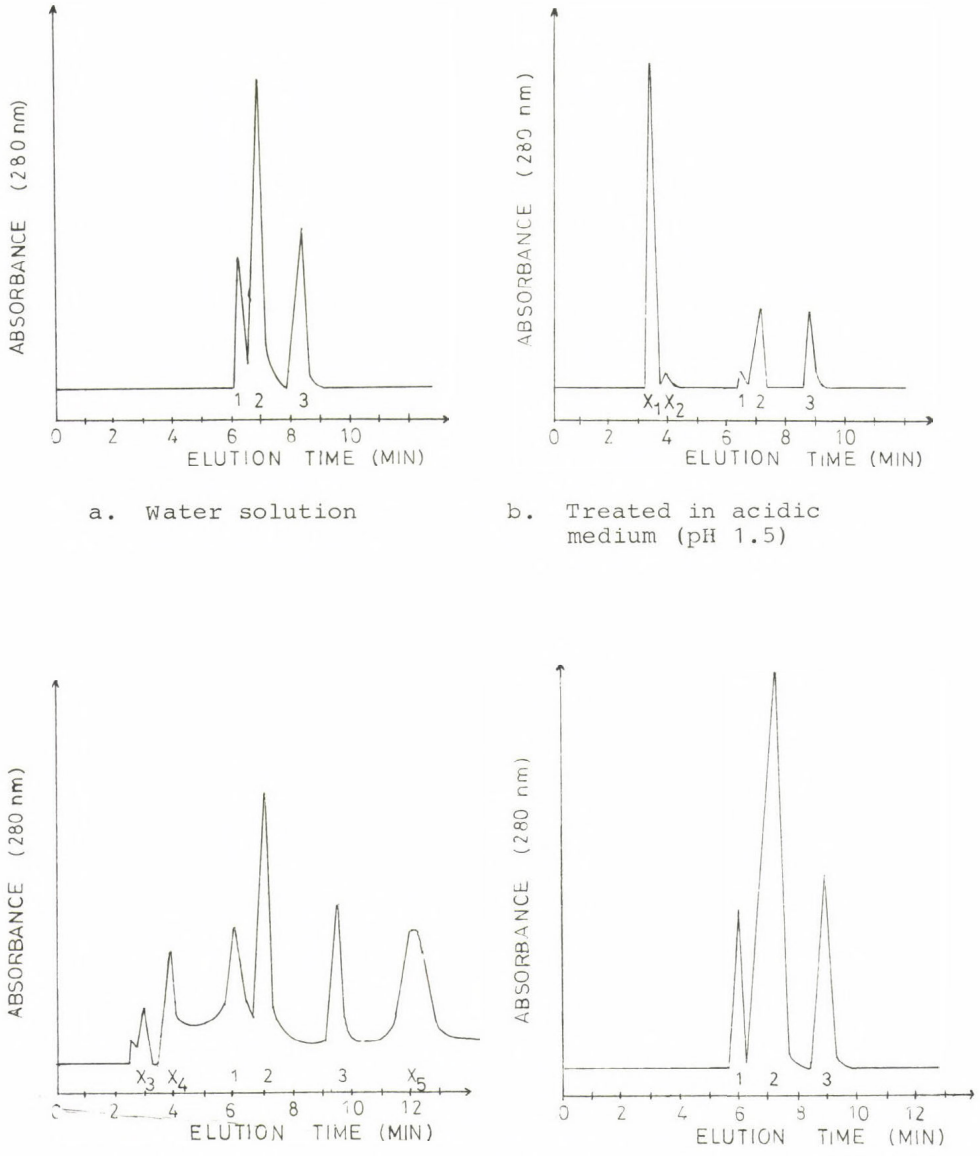

Fig. 1. Comparison of the chromatograms obtained from pure reference samples
1: convicine; 2: vicine; 3: internal standard (sulfaguanidine); x_1-x_5 unknown breakdown products. Injection volume was 20 µl; flow-rate, 1.00 ml/min; chart speed, 1.2 cm/min.
See Methods for further details

interrupted until the suspension cooled to room temperature. Another 30 s long microwave irradiation of the suspension proved to be enough for maximum recovery of the vicine and convicine. Further irradiation already decreased the vicine and convicine content and breakdown products appeared. The supernatant (cf. Methods) was subjected to HPLC analysis.

In order to compare the efficiency of our rapid extraction to that of the extraction described by Lattanzio et al. /9/, the vicine and convicine content of our fababean batch was determined by both methods. The results are summarized in Table 1.

Table 1. Comparison of the present extraction method with that described by Lattanzio et al. /9/

Mode of extraction	Vicine in fababean (% dry weight)	Convicine	Total time needed
Methanol/Water Soxhlet-extraction Lattanzio et al.	0.56 $\pm$ 0.01	0.22 $\pm$ 0.01	3 h
Methanol/Water extraction with 2 x 30 seconds microwave irradiation	0.67 $\pm$ 0.01	0.27 $\pm$ 0.01	<1 h

The standard deviation ($\pm$ values) was calculated from 12 injections of six independently prepared samples.

According to the data in Table 1 our rapid extraction method yielded about 20% higher recovery of vicine and convicine from the beans.

In order to check the possible decomposition of these toxic glucosides during the extraction process described, the methanol:water solution of the pure reference vicine and convicine was also subjected twice to 30 s long microwave irradiation. No loss or degradation of the vicine and convicine was observed (cf. Fig 1a. and Fig. 1d).

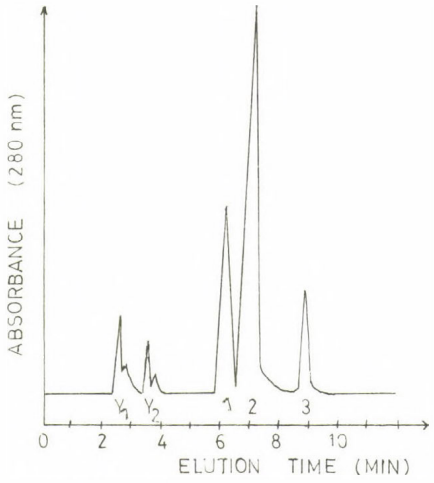

Fig. 2. Chromatographic profile of the untreated bean sample by the present extraction method
1: convicine; 2: vicine; 3: internal standard (sulfaguanidine); y_1 and y_2 unknown compounds extracted from the beans.
Conditions were as given in Fig. 1. or under Methods

Other compounds extracted from fababeans do not disturb the HPLC analysis as they are well separated from vicine and convicine (Fig. 2.). The use of any organic modifier in the eluent which would reduce the retention times of vicine and convicine and consequently the analysis time is detrimental to the good separation presented in Fig. 2.

The microwave extraction method described above and the HPLC technique give a rapid and reliable way for the quantitative determination of vicine and convicine in fababeans. Because of the sensitivity and rapidity of the method it can be applied to test the effects of various technological processings performed on fababeans with the view of reducing the toxic glucoside content.

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SEPARATION AND DETERMINATION OF PHOSPHATIDYLCHOLINE SPECIES AS DIACYLGLYCEROL NAPHTHYLURETHANES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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According to their physiological function different subcellular membranous systems show a different but characteristic acyl chain pattern of membranephospholipids especially phosphatidylcholine (1).

Two major pathways exist in the cell for the generation of this specific species pattern: the de novo pathway for the complete synthesis of phosphatidylcholine and the deacylation reacylation cycle (2-4).

A premise to a successful research in this field is a certain method for the fractionation and determination of phosphatidylcholine species in the micro range. The classic method, first described by van Golde and van Deenen (5), using thin-layer chromatography on $AgNO_3$-impregnated silica gel plates is too insensitive for this purpose. Another field of application using such micro method is the investigation of bioptic specimen described by us in progressive muscular dystrophy (6). We have developed a method for the separation and quantitative estimation of the sn-1.2-diacylglycerol moieties by HPLC after derivatization with α-naphthylisocyanate (7). Diacylglycerol species were formed from the corresponding phosphatidylcholine species by treatment with phospholipase C.

For the separation of the naphthylurethanes obtained a Hewlett-Packard chromatographic column (200 x 4.6 mm) packed with Lichrosorb RP 18 (5 µm) was used.

Detection could be achieved either by UV-absorption at 290 nm or by fluorescence. Using fluorescence detection of the

separated peaks (ex. 280 nm, em. 360 nm) about 10 pmol of a single diacylglycerol species can be detected.

The quantification was based on 1,2-distearoyl-glycerol as the internal standard.

This diacylglycerol can be used because it is not present in our biological samples. The first attempts for estimating a diacylglycerol pattern by our method including recovery experiments and the control of our species by parallel gas chromatographic analysis of the fatty acids in the single peaks was made from diacylglycerols generated by phospholypase C treatment of phosphatidylcholine from rat liver microsomes (7). It is the aim of this study to estimate the phosphatidylcholine pattern in microsomes of different organs, mainly lung.

In lung disaturated species especially dipalmitoyl phosphatidylcholine occur in relatively large quantities. This substance is considered to play an important role in determining the surface tension lowering properties of pulmonary surfactant (8); the measuring of its synthesis and consumption seemed to be of importance for the investigation of diacylglycerol generating and consuming reactions. Unfortunately there was no separation between the 16/16 and the 16/18:1 species in the acetonitrile-water solvent system which was used in our first experiments. Therefore, we tried to use other solvent systems for the separation of our compounds. We found that dipalmitoyl glycerol is clearly separated from the other species by the use of methanol-water as the solvent (see Fig. 2).

A complete separation of all species could not be achieved either in the acetonitrile system or in methanol. Also in the methanol system not all of the peaks represent single diacylglycerol species (see Table 1).

For the identification of the HPLC-separated species we collected the peaks from at least three runs and determined the fatty acid composition of each peak by gas chromatographic analysis of the fatty acid methyl ester. Nevertheless, the exact composition of some minor peaks, especially if they are not clearly separated from the major ones, could not be clearly identified. The chromatograms of diacylglycerol naphthylure-

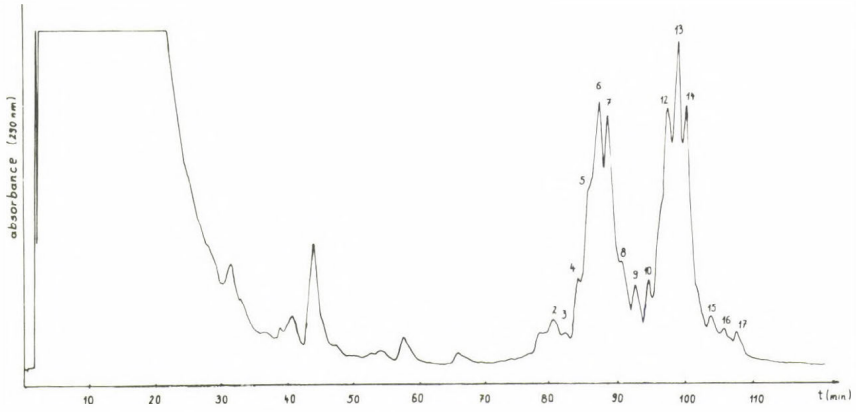

Fig. 1. HPLC separation of diacylglycerol naphthylurethanes from phosphatidylcholine of rat liver microsomes. Peaks are numbered in sequence of elution time and are listed in Table 1

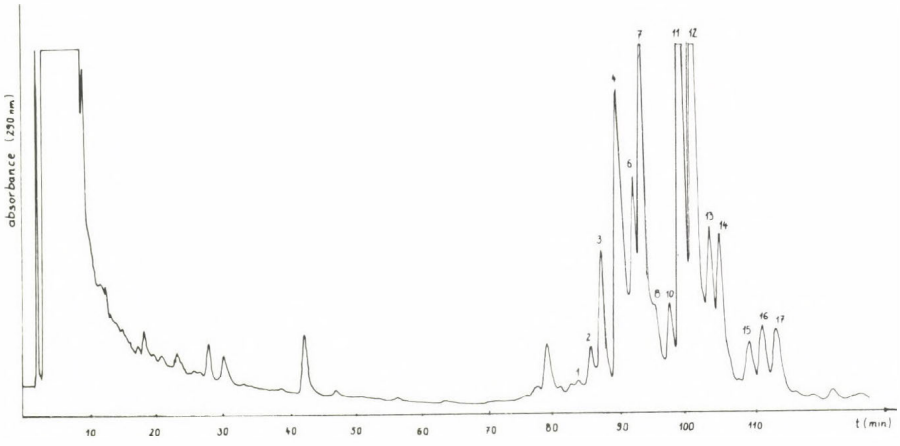

Fig. 2. HPLC separation of diacylglycerol naphthylurethanes from phosphatidylcholine of rat lung microsomes

thanes from phosphatidylcholine of microsomes from rat liver, lung and muscle are demonstrated in Figs 1-3.

As shown in Table 1 there were great differences in the species distribution between these three organs.

Table 1. Distribution of molecular species in phosphatidylcholine from microsomes of rat liver, lung and muscle

Peak number	Molecular species	Percentage composition liver	lung	muscle
1	16:1/18:2	-	-	0.5
2	16/20:5	0.2	-	1.3
3	18:2/18:2	-	-	2.2
4	16/16:1	2.0	12.9	1.4
	18:2/20:4			
5	16/22:6	21.5	-	4.7
6	16/20:4		7.0	11.4
7	16/18:2	13.7	13.1	53.2*
8	16:1/18:1	3.6	-	-
9	18/20:5	2.8	-	-
10	16/20:3	2.3	2.2	4.5
	17/18:2			
11	16/16	-	23.3	3.4
12	16/18:1	17.2	18.1	7.2
	18/22:6			
13	18/20:4	15.9	5.3	2.9
14	18/18:2	14.3	5.2	5.4
15	18/20:3	2.0	1.6	0.9
16	16/18	1.5	2.3	0.4
17	18/18:1	1.3	2.7	0.6

*with 16/22:5 as the minor component

These results agree with data in the literature (9, 10) obtained with thin-layer chromatography. We think that the higher sensitivity of our method as well as the increased efficiency using HPLC give a better approach to the problems involved in the biosynthesis of phosphatidylcholine than conventional methods.

As shown in Figs 1-3 the diacylglycerol moieties of phosphatidylcholine of the investigated tissues were fractionated into17 peaks representing 20 molecular species.

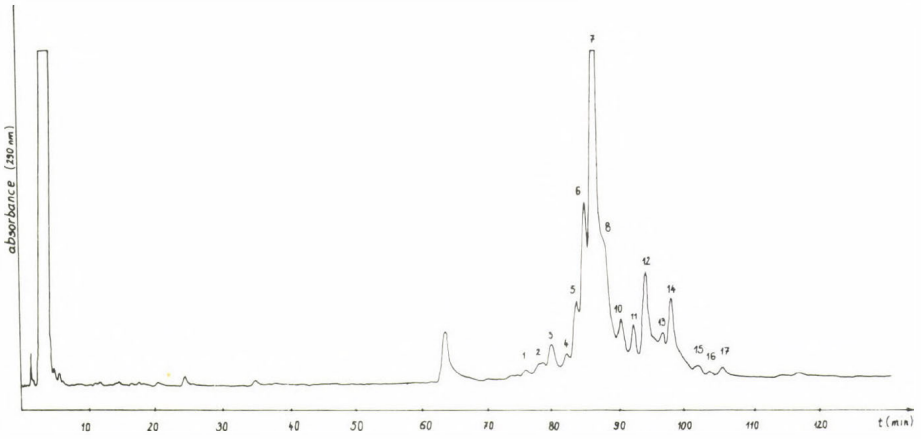

Fig. 3. HPLC separation of diacylglycerol naphthylurethanes from phosphatidylcholine of rat muscle microsomes

Another advantage of our method is the possibility of using radioactive labelled compounds. By collecting the separated peaks the measurement of their radioactivity by liquid scintillation counting is possible.

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RELATIONSHIP BETWEEN HPLC RETENTION AND BIOLOGICAL ACTIVITY OF NITROGEN-BRIDGED COMPOUNDS

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ABSTRACT

A good correlation was found between the partition coefficient and the retention data (log K and log k') for certain types of nitrogen-bridged compounds. Accordingly, the relationship between the biological activity and these two physical parameters was studied, and a good correlation was again demonstrated.

INTRODUCTION

For several years we have been investigating the HPLC of nitrogen-bridged compounds /1-3/, the relationships between the chemical structure, the partition coefficient and the HPLC behavior being extensively studied. Our results clearly show that the partition coefficient (log K) is directly related to the capacity factor (log k'). Although similar relationships have been established by various authors for different types of compounds, the relationship between the biological activity and HPLC behaviour has received only limited attention /4, 5/. In the present paper proof of the existence of such a relationship is supplied. As a logical consequence, HPLC may be used as a method for the prediction of biological activity instead of using the log K value, the determination of which involves a more laborious and time-consuming procedure. The selected ten

model substances (Table 1) are pyridopyrimidine derivatives and have antiasthmatic activity.

These compounds were synthesized during a systematic research program to elucidate pharmacological profiles and stucture - activity relationships among nitrogen-bridged compounds /6-8/.

EXPERIMENTAL

Chemicals. All of the model substances (Table 1) were synthesized in our laboratory. Their identification and quality control were performed by melting point determination and chromatography.

Reagents and solvents were of analytical grade (Merck) and were used without further purification.

Equipment and chromatographic conditions. Chromatography was performed with a Liquochrom chromatograph Model 2010 (Labor MIM, Budapest), on an Ultrasphere ODS column (Beckman) (250x4.6 mm). The column was prepacked with 5 μm particles. A variable-wavelength detector was used. The column effluent was monitored at various wavelengths in the interval of 270-340 nm. Methanol-water mixtures of various ratios (80:20, 70:30, 60:40) were used as the mobile phase. For the regression calculation, the capacity factors obtained with the 70:30 methanol-water mixture were used. The flow rate was 0.7 ml/min. All experiments were run at room temperature (25°C). The tested compounds were dissolved in methanol to give a concentration of 100 μg/ml. 20 μl aliquots of these solutions were injected.

The procedure for partition coefficient determination has been described previously /9/.

Konzett test /10/. Principle of the method: the aim of this test is to demonstrate the bronchospasmolytic activity of the tested compounds on bronchial spasms produced by intravenous injection of 5-hydroxytryptamine (serotonin), histamine or acetylcholine every 5 minutes. Material and methods: Hartley guinea-pigs of either sex, weighing 250 to 500 g, were anaesthetized,curarized and artificially ventilated. The two

Table 1. Structure of model substances

Number of compounds		Number of compounds	
1.	n = 1	13.	n = 1
2.	n = 2	14.	n = 2
3.	n = 3	15.	n = 3
4.	n = 4	16.	n = 4
2.a	C_6 Me		
2.b	C_7 Me		
2.c	C_8 Me		
2.d	C_9 Me		
		17.	n = 1
		18.	n = 2
		19.	n = 3
		20.	n = 4
		17.a	C_6 Me
5.	n = 1	17.b	C_7 Me
6.	n = 2	17.c	C_8 Me
7.	n = 3	17.d	C_9 Me
8.	n = 4	18.a	C_6 Me
5.a	C_6 Me	18.b	C_7 Me
6.a	C_6 Me	18.c	C_8 Me
6.b	C_7 Me	18.d	C_9 Me
6.c	C_8 Me		
6.d	C_9 Me		
		21.	n = 1
		22.	n = 2
		23.	n = 3
9.	n = 1	24.	n = 4
10.	n = 2	21.a	C_6 Me
11.	n = 3	21.b	C_{12}Me
12.	n = 4	23.a	C_{12}Me

jugular veins were catheterized, one being used for curarization and administration of the drugs, and the other for the injection of the spasmogenic agent. The intratracheal pressure was measured with a transducer connected to a tracheal cannula and recorded on a Sanborn polygraph. For each of the three spasmogenic substances, the bronchospasmolitic activity was assayed on four guinea-pigs. The tested compound was injected intravenously into the same animal every 15 minutes, in progressive doses. The activity was expressed as the percentage change versus the control spasms. If a dose of the drug reached at least -50%, the regression line of the percentage versus the dose was computed in a semilogarithmic system. The ED_{50} was then calculated.

RESULTS AND DISCUSSION

The numerical biological activity data obtained from the Konzett test are shown in Table 2. The ED_{50} values of the model compounds compared to that of theophylline point to the potential antiasthmatic character of the tested compounds. As expected, the correlation between the responses (ED_{50} values) provoked by the three different agents in the presence of the

Table 2. The result of Konzett test

No	Biological activity data log ED_{50}			
	5-HT	Hist.		AcCh
		obs.	pred.	
1	-1.916	-1.663	-1.683	-2.00
2	-1.38202	-1.403	-1.412	-1.3747
2.a	-1.352	-1.276	-1.176	-1.7512
5	-1.474	-1.382	-1.370	-1.708
5.a	-1.6702	-1.294	-1.231	-1.8727
17.a	-1.049	-1.009	-1.016	-1.2601
17.c	-1.13988	-1.06819	-1.119	-1.196
17.d	-0.88366	-1.0607	-1.086	-1.1072
18.d	-1.19312	-0.843	-0.832	-1.196
22	-1.1818	-1.045	-1.119	-1.493
Theophylline	1.146	1.146		1.342

Table 3. Correlation between log K and log k' values

No	log k'	log K	
		obs.	pred.
1	-0.440	0.220	0.1978
2	-0.2284	0.7070	0.81002
2.a	-0.0442	1.2066	1.3429
2.b	-0.12963	1.1933	1.096
2.c	-0.02896	1.2049	1.387
3	-0.1123	1.251	1.146
4	0.027152	1.613	1.5494
5	-0.196	0.822	0.9038
5.a	-0.087	1.222	1.2191
6	-0.0414	1.172	1.351
6.a	0.09970	1.6741	1.759
6.c	0.07684	1.6742	1.693
7	0.1044	1.729	1.7729
8	0.2253	2.129	2.1226
9	-0.0639	1.239	1.2859
10	0.05270	1.599	1.6233
11	0.2808	2.178	2.2832
12	0.27949	2.429	2.2794
15	0.30797	2.409	2.362
17	-0.11197	1.188	1.1769
17.a	0.0801	1.7730	1.7025
17.b	0.00	1.6143	1.471
17.c	0.00	1.6475	1.471
17.d	0.0260	1.7114	1.546
18	0.05551	1.633	1.6314
18.a	0.224	1.9073	2.1189
18.b	0.18988	2.068	2.020
18.c	0.16185	1.8604	1.939
20	0.2939	2.290	2.321
21.b	0.01911	1.591	1.526
22	0.00	1.485	1.471
23	0.1344	1.977	1.8596
23.a	0.338	2.439	2.4487

test compounds is rather poor. Furthermore, the ED_{50} values for the histamine and serotonin responses coincide better than the values of the histamine-acetylcholine correlation. Table 3 shows data on the correlation between log K and log k' for 33 compounds. Table 4 and Figs 1 and 2 also demonstrate close correlations between the histamine test values (i.e. biological activity) and log K and log k'. The correlations between these physical parameters and the results of the serotonin and acetylcholine tests are significantly poorer.

Table 4. Statistics of correlations

		Statistics	
		r	F
log K/log ED_{50}			
	5-HT	0.8399	19.2
	Hist.	0.9768	166.6
	AcCh	0.7499	10.3
	n = 10		
log K/log k'			
		0.9797	740
	n = 33		
log k'/log ED_{50}			
	5-HT	0.78356	12.7
	Hist.	0.9774	171.0
	AcCh	0.7101	8.1
	n = 10		

5-HT 5-hydroxytryptamine (serotonin)

Hist. Histamine

AcCh Acetylcholine

r log ED_{50} 5-HT-Hist. = 0.834

r log ED_{50} Hist-AcCH = 0.788

r log ED_{50} 5-HT-AcCh = 0.904

n = 10

The results lead to the conclusion that the HPLC retention (capacity factor) in a suitable chromatographic system can correlate with the biological activity in all cases when a linear relationship exists between the capacity factor and partition coefficient. This promises new possibilities in the application of HPLC in the practice of drug research.

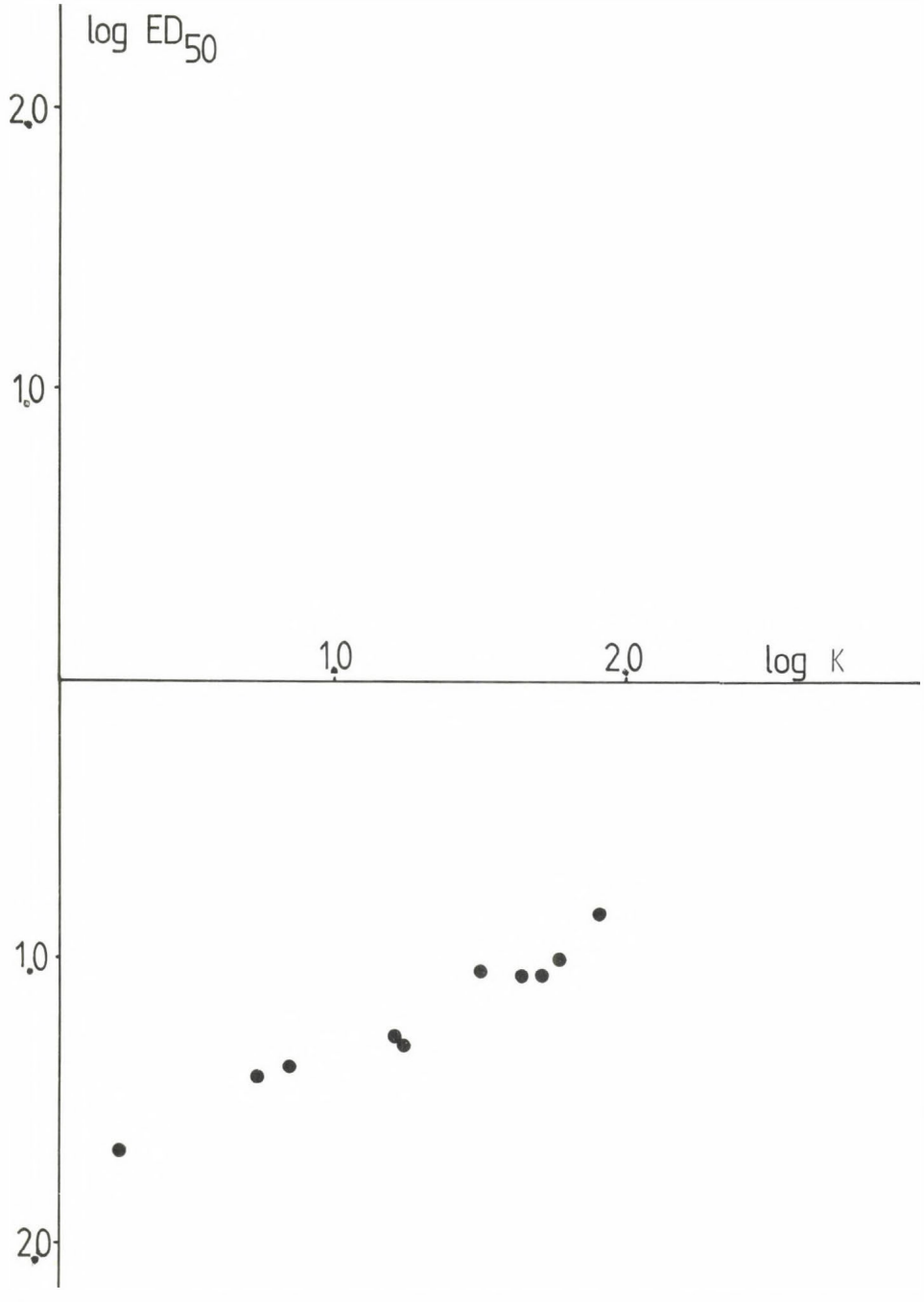

Fig. 1. Correlation between the log K values and log ED_{50} (Hist.)

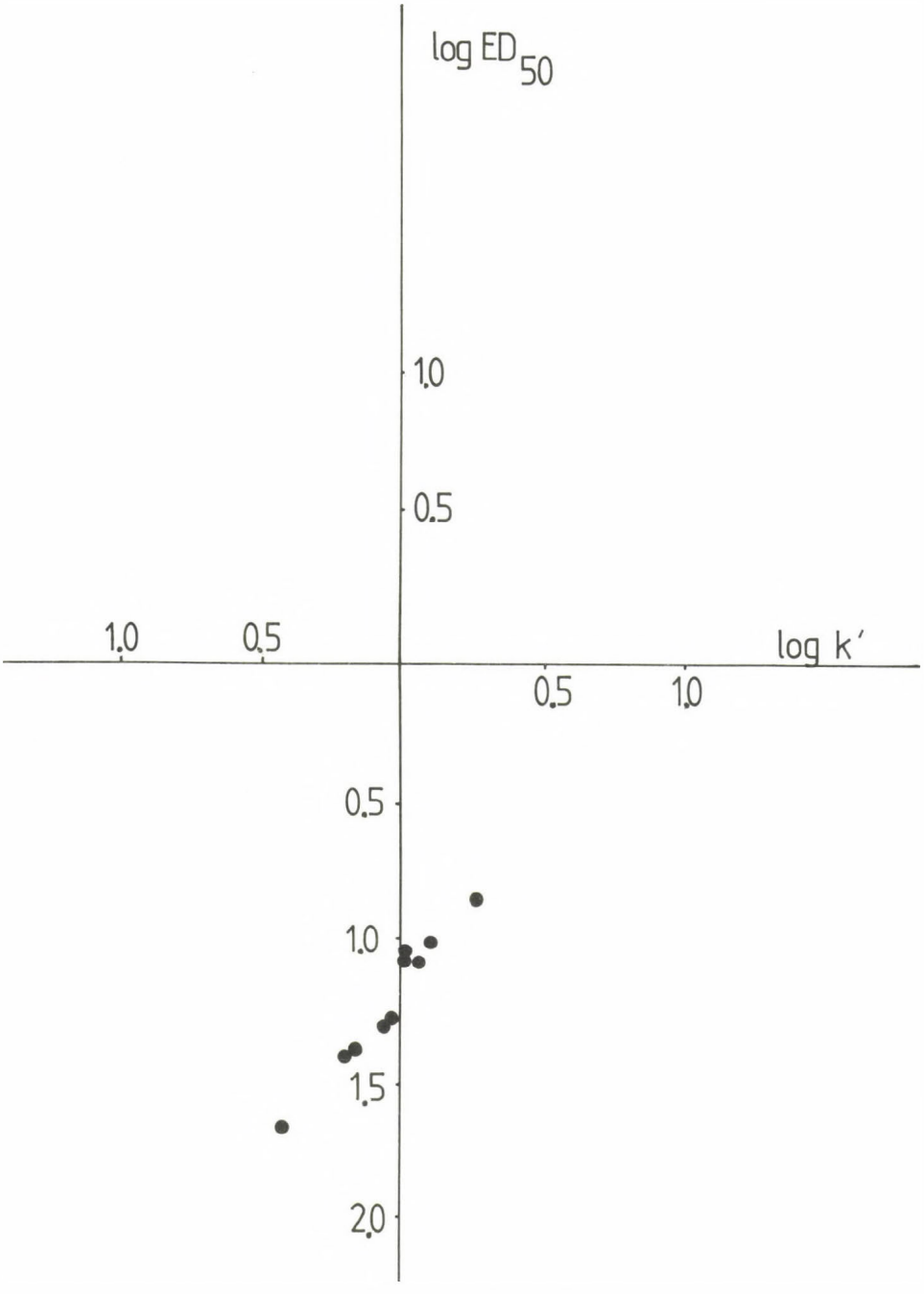

Fig. 2. Correlation between the log k' values and log ED_{50} (Hist.)

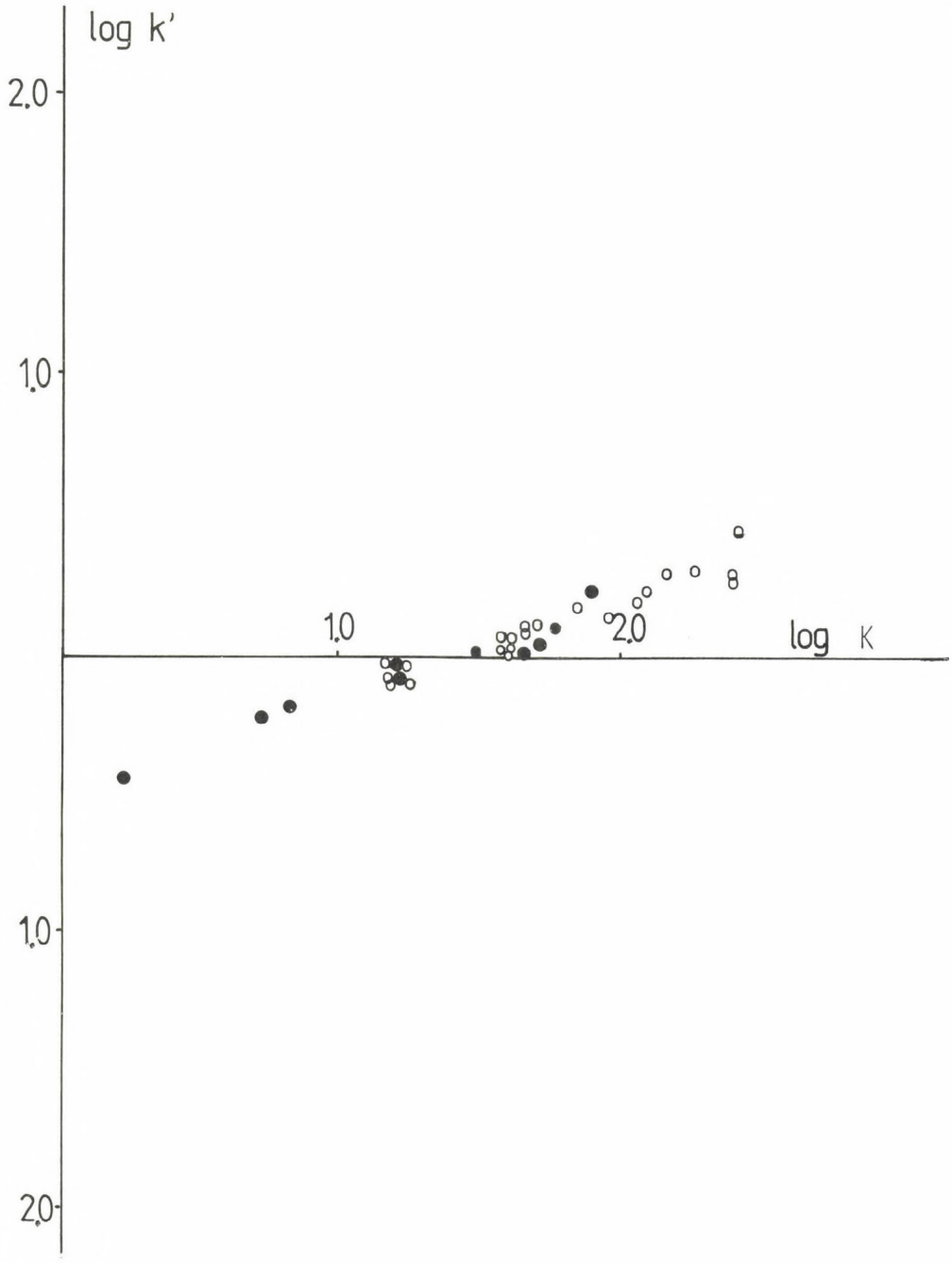

Fig. 3. Correlation between the log k' values and the log K values

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H. Kalász and L.S. Ettre (Eds)

ION-PAIR TLC OF QUATERNARY AMMONIUM IONS WITH HYDROPHILIC ANIONS ON SILICA

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TLC of quaternary ammonium ions on unsubstituted silica (silica gel G Merck) with anions such as chloride, bromide, nitrate and perchlorate in aqueous mobile phases (1, 2), containing solvents such as acetone, dioxane or ethanol, can be regulated by the concentration and the radius of the counter-ion (3), the solubility parameter of the solvent (4), the charge and the degree of substitution of the sample ion (5).

The relationship between the R_M value of the ammonium ions and the concentration of the counter-ion is regulated by the equation:

$$R_M = n \cdot \lg c_A^{-1} - n \cdot \lg c_o^{-1}$$

n = charge of the quaternary ammonium ion

c_A = concentration of the counter-ion (mol/L)

$c_o = c_A$ for $R_M = 0$

(stationary phase: silica gel G (Merck);
mobile phase: acetone/aqueous solutions of the counter-ions: chloride up to 0,5 mol/L, bromide up to 0,4 mol/L, nitrate up to 0,3 mol/L, perchlorate up to 0,2 mol/L)

The relationship between the R_M value of the sample ions and the radius of the counter-ions is regulated by the equation:

$$R_M = \frac{n}{2r_A} - \frac{n}{2r_o}$$

r_A = radius of the counter-ion

r_o = radius for $R_M = 0$

(mobile phase: acetone/aqueous solutions of chloride, bromide, iodide, and perchlorate (0,2 mol/L))

The influence of the solvent on the R_f value of the sample ions is shown in Table 1.

Table 1. R_f values in a mobile phase with 1 volume of HCl (1 mol/L) and 1 volume of solvent

Ammonium ion	R_f value with solvent			
	A	B	C	D
choline	0,72	0,70	0,65	0,46
carbachol	0,71	0,69	0,65	0,45
methyloxamethonium	0,37	0,37	0,33	0,11
pentamethonium	0,42	0,40	0,35	0,12
suxamethonium	0,49	0,47	0,37	0,20
gallamine	0,24	0,22	0,22	0,05

A = 1:1 acetone/tetrahydrofurane;
B = acetone;
C = dioxane;
D = ethanol

Table 2. R_f values in acetone/water with different counter-ions with increasing concentration

Sample ion	counter-ion	concentration of the counter-ion (mol/L)	R_f value
hexamethonium	ClO_4^-	0,10	0,28
		0,15	0,45
		0,20	0,60
	NO_3^-	0,15	0,15
		0,20	0,22
		0,30	0,40
pentamethonium	Br^-	0,20	0,15
		0,30	0,30
		0,40	0,43
	Cl^-	0,20	0,09
		0,30	0,23
		0,40	0,32
		0,50	0,40

Table 3. R_f values of quaternary ammonium ions in acetone/aqueous solution of counter-ion (0,2 mol/L)

Sample ion	counter-ion	R_f value
carbachol	Cl^-	0,55
	Br^-	0,60
	I^-	0,74
suxamethonium	Cl^-	0,10
	Br^-	0,22
	I^-	0,47
	ClO_4^-	0,60
pentamethonium	Cl^-	0,09
	Br^-	0,15
	I^-	0,40
	ClO_4^-	0,52

The selectivity of a mobile phase with ethanol and chloride solution is twice of that with acetone (6).

The retention of quaternary ammonium ions on unsubstituted silica with inorganic anions in aqueous mobile phases decreases with

- increasing concentration of the counter-ion (until saturation)
- increasing radius of the counter-ion
- decreasing solubility parameter of the solvent in the mobile phase
- decreasing charge of the sample ion
- increasing number of CH_2-groups of the ammonium ion.

The R_f value of bisquaternary ammonium ions changes, approximately, linearly with the concentration of the counter-ion (until saturation) (Table 2, for example).

The R_f value of quaternary ammonium ions changes, approximately, linearly with the radius of the counter-ion (Table 3 for example).

The mechanism of TLC on silica with

- methanol only is
  - ion-exchange for aprotic ammonium ions
  - ion suppression for protic ammonium ions
- methanol/HCl is
  - ion-pair chromatography for all N-compounds.

Therefore separations of protic and aprotic ammonium ions are possible by two-dimensional TLC.

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BIOLOGICAL DETECTORS IN LIQUID CHROMATOGRAPHY

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The general principle of biodetection has been further developed and the basic application areas described by Gróf et al. in our laboratory /1/. Working with this method we have previously reported that various isolated organ preparations long in routine use in a wide spectrum of biological researches can also be successfully applied as chromatographic detectors, specifically called biological detectors, or simply biodetectors /2, 3/. The compatibility of these two types of detectors is based on the fact that biological detectors share a fair number of the important properties of up-to-date conventional chromatographic detectors. Due to the remarkable sensitivity as well as specificity of biodetectors chromatographers applying both conventional and biological detectors may obtain important excess information mainly on the chemical structure but also on the possible physiological role of unknown endogenous compounds with biological interest simply by registering their biological activities with the aid of one, or a whole set of appropriately selected biological detectors.

In our previous reports /1-3/ the applicability of biological detectors have been demonstrated in experiments where ultrafiltered sera of healthy persons and schizophrenic patients, as well as acidic acetone extract of pregnant sow ovaries were fractionated on a Sephadex G-25 (SG-25) column. In those experiments UV absorbance was monitored at 254 nm, whereas biological activities of individual fractions were determined by using isolated mouse vas deferens and rat uterus strip /2, 3/. However, in none of the experiments cited above were the bio-

logical detectors connected directly to the chromatographic column. Instead, biological activity of separated fractions were determined by one-by-one injection of separately collected chromatographic fractions into the detector cell (off-line) detection /2, 3/. As the detector cell used was not a through-flow-type, it allowed only a periodical flushing of the biodetector with a solution of physiological composition, e.g. a Krebs-Ringer solution in the referred cases /2, 3/.

Occasionally, however, organ preparations with intensive metabolism may also be desirable to be applied as a biodetector. By using organ preparations of this type, keeping the well being and especially the good functional state of the isolated organ might necessitate a continuous washing of the detector cell with appropriate physiological solution. Without such measures, isolated organs with intensive metabolism would rapidly deteriorate, thereby preventing their use as biological detector.

To overcome this difficulty, it is necessary to use a flow-through-type detector cell allowing a continuous flushing of the isolated organ placed in the detector cell. Such a flow-through-type detector cell allows not only the use of isolated organs with intensive metabolism as a biodetector, but also to directly connect the detector cells to the chromatographic column. In such an arrangement, possible biological activities of the components successively eluted from the column may be directly and continuously registered (on-line detector).

In our paper, operational principle of the on-line biodetectors will be outlined and their applicability in the practice demonstrated by presenting experimental data collected in our laboratory.

ON-LINE BIOLOGICAL DETECTORS

The principal component of on-line biodetector systems is the flow-through-type detector cell which, however, can be operated both in an off-line and on-line mode. The detector cell shown in Fig. 1. is a flow-through-type thermostated glass

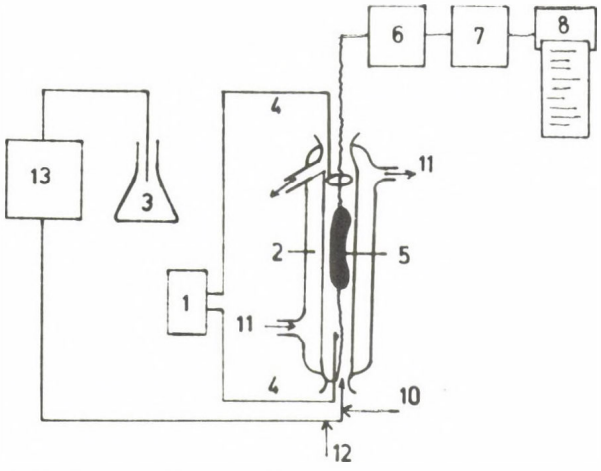

Fig. 1. Flow-through-type detector cell allowing off-line operation of a biodetector. 1: electrostimulator; 2: detector cell; 3: washing solution; 4: electrodes; 5: isolated organ; 6: extensometer; 7: bioforcemeter; 8: recorder; 10: carbogen gas inlet; 11: thermostat; 12: site of sample injecting; 13: peristaltic pump

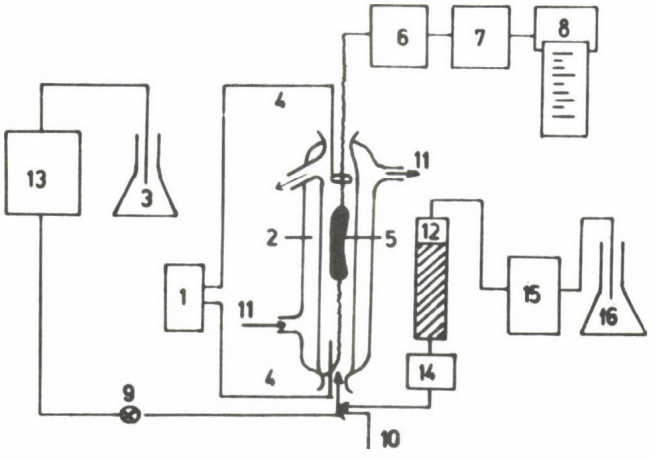

Fig. 2. Flow-through-type detector allowing on-line operation of biodetectors. 1: electrostimulator; 2: detector cell; 3: auxiliary solution; 4: electrodes; 5: isolated organ; 6: extensometer; 7: bioforcemeter; 8: recorder; 9: valve; 10: carbogen gas inlet (5% CO_2 + 95% O_2); 11: thermostat inlet and outlet; 12: chromatographic column; 13: peristaltic pump; 14: UV detector; 15: peristaltic pump; 16: reservoir for the eluent

vessel of 1 ml volume. The electrodes of an electrostimulator are fixed at the top and the bottom of the cell. Continuous flow of the washing solution through the detector cell is ascertained by a peristaltic pump. Biological responses (e.g. contractions) of isolated organs used as the detector are registered by an appropriate recording instrument (e.g. a potentiometric recorder connected to a Bioforcemeter).

If a discontinuous registration of biological activity elicited by components in the separately collected fractions is aimed, aliquots of each fraction snould be injected one-by-one into the detector. In this case the system operates in an off-line manner. As in this system the organ preparation is continuously flushed with a physiological solution throughout the whole experiment, this experimental set-up allows the application of isolated organs of intensive metabolism as biological detectors.

The system shown in Fig. 2. represents a variety of the flow-through-type system which, apart from securing a continuous flushing of the detector cell with a physiological solution, may also be mounted with a biodetector connected directly to the chromatographic column, i.e., with an on-line detector. If the flow rate of the eluent is high enough to prevent the accumulation of metabolic products of toxic character in the surroundings of the biodetector, and the composition of the effluent is appropriate (identical with, or near to the composition of the feeding solution of the isolated organ) the auxiliary pump (marked with 13 in Fig. 2.) does not need to work. In this case, composition of the effluent reaching the biodetector remains unchanged.

If the flow rate of the effluent is lower than desirable, an auxiliary solution should be added to the effluent (the composition of which should be identical with that of the chromatographic eluent) in a quantity which augments the flow rate of the effluent to the desired value. In order to accomplish this, the system should be mounted with an auxiliary pump.

If the composition of the chromatographic eluent deviates from that of the bathing solution of the isolated organ to an extent that endangers proper functioning of the biodetector,

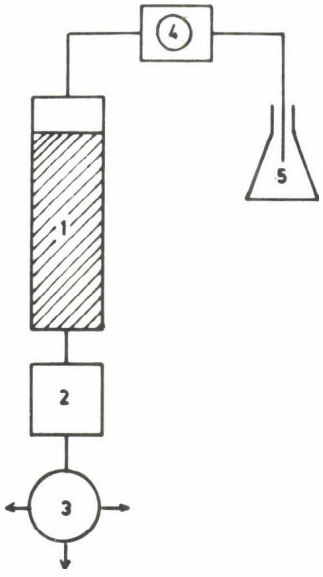

Fig. 3. Distribution head. 1: chromatographic column; 2: UV detector; 3: distribution head: 4: peristaltic pump; 5: eluent reservoir; 6: to biological detectors and to collector

the effluent has to be changed post-column to a solution tolerable for the isolated organ by the addition of an auxiliary solution of appropriate composition to the effluent.

Occasionally, both the quantity and quality of collectable information may be significantly enhanced by placing a conventional detector (e.g. a UV detector) between the chromatographic column and the biological detector (Fig. 2.). By mounting the system with an appropriately constructed distribution head (Fig. 3.), simultaneous determination of multiple biological activities initiated by various effluent components can also be accomplished by using an on-line system.

This necessitates the simultaneous use of several isolated organ preparations in parallel connection, each displaying a biological response elicitable by specific signals represented by various substances in the effluent.

DEMONSTRATION OF THE APPLICABILITY OF BIOLOGICAL DETECTORS

We have previously reported /2, 3/ on the successful fractionation of an acidic acetone extract from pregnant sow ovaries by using an SG-25 column under the following conditions: V_t = 84.4 ml; eluent = 0.9% NaCl, pH = 5.0; fraction volume = = 5.0 ml; sample volume = 5.0 ml; flow rate = 0.5 ml $min.^{-1}$ UV absorbance was monitored at 254 nm (LKB Uvicord I), whereas biological activity of individually collected fractions was detected on a rat uterus strip used as an off-line biodetector. The detector cell was filled up with a Krebs-Ringer solution, the composition of which, together with the parameters of the electric stimulation applied, were described elsewhere /2, 3, 4/. By using this system, 13 components could be detected in the effluent fluid, with distinct actions on the rat uterus strip.

In the present study, SG-25 fractionation of a similar sow ovary extract was repeated under chromatographic conditions identical with those applied previously. However, the biological detector operated as an off-line detector in the former experiment, was now substituted by an on-line detector, that is the through-flow-type cell was connected directly to the chromatographic column (Fig. 2.). In addition, since the composition of the effluent applied in this study did not secure an optimal condition needed for proper functioning of the biodetector, the effluent fluid was completed with an auxiliary solution of appropriate composition to secure a fluid composition tolerable for the biodetector. Biological activities of effluent components registered by this on-line detector system /5/ were in good agreement with those registered by the formerly applied off-line detector, both in terms of the number and quality of biological responses elicited /2, 3, 4/.

In another series of experiments, amniotic fluid from healthy women sampled at the 20th week of their pregnancy were centrifuged at 4000 rpm for 20 min at room temperature. The supernatant was fractionated on an SG-25 column under the following conditions: V_t = 80.4 ml; eluent = Krebs-Ringer solution; with a composition described previously /6/; flow rate = 0.5 ml min^{-1}

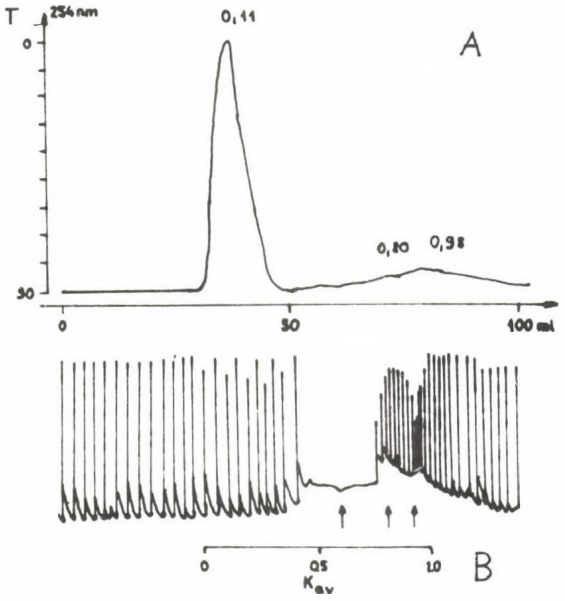

Fig. 4. A: chromatographic profile of amniotic fluid separated on the SG-25 column (chromatographic parameters: see text). Abscissa: fraction volume; ordinate: transmittance at 254 nm. B: Biodetection of effluent containing amniotic fluid components separated on the SG-25 column referred to in part A of the figure, using rat uterus strip as the biological detector. Abscissa: K_{av} values; ordinate: amplitude of contractions.

sample volume = 5.0 ml. Also the parameters of electric stimulation were identical to those specified in the previous experiment. UV absorbance was monitored at 254 nm (LKB Uvicord I). Biological activity was detected by the aid of a rat uterus strip preparation operated as an on-line detector. Since in this study, a Krebs-Ringer solution was applied as the eluent and its flow rate was high enough, administration of an auxiliary solution and operation of an auxiliary pump proved be unnecessary. Continuously registered biological activities and the chromatographic profile obtained at 254 nm are shown in Fig. 4. By the sole use of UV detection at 254 nm, three components could be detected in the investigated sample: one major component (K_{av} = 0.11); and two minor components (K_{av} = 0.30

and 0.98), respectively. Using the biological detector, three fractions could also be detected which affected the contractions of the rat uterus strip differently: the fraction with a K_{av} value of 0.63 inhibited the contractions, the fraction with a K_{av} value of 0.82 increased both the tone and the frequency of the contractions, and the fraction with a K_{av} value of 0.93 decreased the amplitude but increased the frequency of the contractions of the biodetector used. Comparison of the chromatographic profile obtained by monitoring the UV absorbance and the profile obtained by biological detection convincingly demonstrated the applicability of the biological detector and especially its capability of providing additional information in comparison to those obtainable by conventional detection alone. UV detection revealed only the presence of one major component (K_{av} = 0.11) while the other two components (K_{av} = 0.80 and 0.98) appeared to be negligible. The biological detector also registered three fractions, but it also revealed their biological activities on the rat uterus strip detector. It is worth mentioning that the components displaying the most pronounced biological activity could have hardly been detected by the sole use of a UV detector, due to the negligible UV absorbance of the effluent components in question.

DISCUSSION

Results presented both in this and previous reports /2, 3/ have provided evidences that isolated organ preparations may be applied as chromatographic detectors, occasionally with excellent success. Biological detectors meet most criteria of up-to-date conventional detectors. Their sensitivity is high enough and is comparable to that of most conventional detectors (e.g. UV detectors). They respond quickly to changes in effluent composition and their response is proportional to the log concentration of the acting agents in a wide concentration range. In most cases, responses of biological detectors are highly specific. They also have the capability of detecting a compound with characteristic biological activity even in the

occasionally extremely complex matrix of substances displaying similar physico-chemical and chemical properties but dissimilar biological activities.

Biological detectors can be operated in an off-line manner when biological activity of individual fractions obtained by a conventional separation procedure is determined discontinuously by injecting them one-by-one into the detector cell. The use of flow-through-type cells permits not only to apply organ preparations with intensive metabolism as biodetectors, but also to connect biological detectors directly to the chromatographic column and operate them in an on-line manner. On the other hand, completing the system with a properly constructed distribution head allows simultaneous registration of multiple biological activities with the aid of several biological detectors connected parallel to each other. Conventional detector fitted between the chromatographic column and the biological detector may enhance the number of collectable information. On-line biological detection can also be accomplished even in cases when composition of the eluent is aphysiological, by carrying out auxiliary measures.

Components in the effluent which may adversely affect isolated organs should be eliminated prior to the biological detection, a situation when biodetectors can be operated in an off-line manner only. This may occasionally require extra work, but the several advantages offered by a biological detection certainly compensate for it.

SUMMARY

In the present study, the applicability and application principle of appropriately selected isolated organs as chromatographic detectors has been reported. In contrast to our previous publications reporting on biological detectors operated in an off-line manner /2, 3/, the present study was devoted to the description of a flow-through-type detector cell, which is directly attached to the chromatographic column allowing isolated organs to be operated as on-line biological detectors.

The applicability of such detector systems was demonstrated by presenting experiments in which acidic acetone extract of sow ovaries, as well as native amniotic fluid samples derived from healthy pregnants have been fractionated on a SG-25 column, and in addition to measuring its UV absorbance, the effluent was also subjected to a biological detection procedure. Continuously monitored UV absorbance profile and the biological activity profile obtained by using rat uterus strip as a biological detector have been compared. This comparison provided additional evidences supporting the earlier assumption that isolated organ systems can be applied as chromatographic detectors just as well as conventional detectors, simply because these two types of detectors share many common properties such as their sensitivity, their specificity and the rapidity of their responses. It was also demonstrated that biodetectors may be occasionally superior than conventional chromatographic detectors, due to some additional properties possessed by the biodetectors but lacking by conventional detectors.

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SEPARATION OF AMINO ACIDS, POLYPEPTIDES AND NUCLEOTIDES

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AMINO ACID ANALYSIS WITHIN 25 MINUTES USING C_1 AND C_{18} REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY COLUMNS WITH o-PHTHALALDEHYDE PRECOLUMN DERIVATIZATION; APPLICATION FOR BIOLOGICAL SAMPLES

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INTRODUCTION

Amino acid analysis is an important and widely used method in biochemical investigations. The first modern, automatized procedure offering acceptable analysis time for routine analysis was based on ion-exchange chromatography followed by ninhydrin derivatization /1/. Since then many chemical labeling procedures have been developed /2/ which enhance sensitivity. Frequently used fluorescent reagents like fluorescamine /3/ and o-phthalaldehyde (OPA) /4/ permit more sensitive analysis than ninhydrin /5/.

Further development of the chemically bonded reversed-phase HPLC packing materials /6/ allowed more advanced precolumn derivatization methods. As a result of this approach, the analysis time was decreased below 40 min /7/ and the detection limit to about 50-100 femtomole amino acid /8/. Detailed investigations showed that adducts formed between amino acids and OPA /9/ or fluorescamine /10/ are unambiguous only in the case of OPA (Fig. 1.), which makes OPA a well applicable precolumn derivatization agent in amino acid analysis.

Enhanced fluorescence and increased lifetime of OPA-amino acid adducts have been recently reported /11/ using various surfactants in the reaction medium. On the other hand the surfactants are recommended as eluent additives over the critical micelle concentration (CMC) to improve the selectivity of RP-HPLC /12, 13/. With regard to disadvantages of certain hetae-

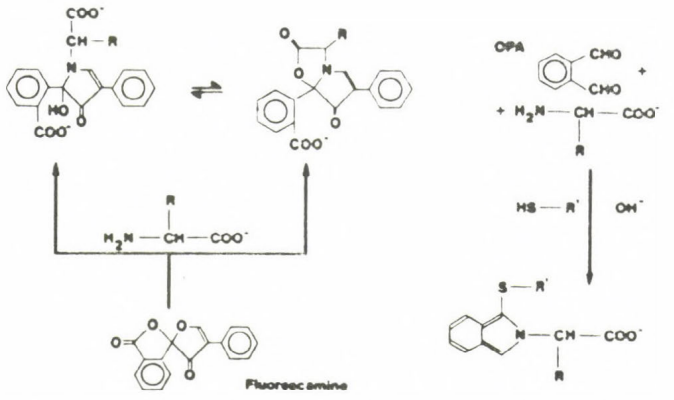

Fig. 1. Reaction of fluorescamine and OPA with amino acids

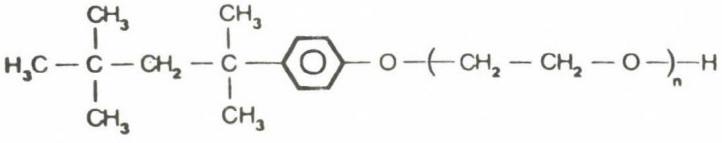

Fig. 2. Chemical structure of Triton X-100 (n = 10)

rons /13, 14/ we have selected Triton X-100 (Fig. 2.) as the modifier agent.

Chemically bonded C_{18} stationary phases are frequently used to separate amino acid derivatives. Unfortunately in many cases difficulties were observed in the separation of Thr-, and Gly-OPA adducts on C_{18} stationary phases without the use of tetrahydrofuran in the eluent /15, 16/.

This report describes a rapid and sensitive method for amino acid analysis using C_1 or C_{18} stationary phases, by a routine HPLC equipment. Analysis time was within 25 min and detection limit at the picomole amino acid level. The amino acids were analysed as their OPA derivatives via a simple and rapid precolumn derivatization, which requires only two minutes.

The short analysis time, the good detection limit, the easy way of sample preparation, the need for small sample size

(15-30 µl), the low background of fluorescent detection make this method especially suitable for the routine analysis of biological samples. Therefore in this paper we also report on the extra- and intracellular amino acid spectrum of the isolated rat liver cell system which is widely used in cellular metabolism studies.

MATERIALS AND METHODS

Instrumentation

HPLC was performed with a Model 2010 Liquid Chromatograph consisting of an OE-312 type pulseless pump, an OE-308 type UV spectrophotometric detector (using a 8 µl flow cell with 10 mm optical path), an OE-320 type injector valve with 20 µl loop and an MP-85 type computing integrator (all from Labor MIM, Budapest, Hungary). The fluorescence of the effluent was monitored by a Model 420 fluorimetric detector (Waters Assoc. Milford, MA., USA), which was connected to the outlet of the UV detector. The chromatograms were recorded either on a one-channel recorder (OH-814/1 type, Radelkisz, Budapest, Hungary), or a two-channel recorder (TZ-4201 type, Laboratorny Pristoe, Prague, Czechoslovakia). A graphically programmed low-pressure solvent delivery pump (PPM 68005, Microtechna, Prague, Czechoslovakia) was used to generate the eluting gradients.

Columns

We used either a prepacked 250 x 4.0 mm I.D. stainless steel column filled with Chromsil C_{18} (6 µm particle size, from Labor MIM, Budapest, Hungary) or a 150 x 4.0 mm I.D. stainless steel column packed in our laboratory according to the manufacturer's instructions with 5 µm particle size SAS-Hypersil (Shandon Southern, England) packing material.

When biological samples were examined, the analytical columns were protected by a 72 x 2.1 mm I.D. type A guard column (Chrompack, The Netherlands).

Derivatization

The procedure of Jones et al. /17/ was slightly modified. Component A consisted of a 2% sodium dodecyl sulphate (SDS) solution in 0.4 M borate buffer (pH 9.5); component B consisted of 50 mg of OPA in 1.25 ml methanol, 50 µl mercaptoethanol, and 11.2 ml of 0.4 M borate buffer; component C consisted of 0.15 M KH_2PO_4 (pH 4.0).

Derivatization procedure: 15-30 µl aliquots of the sample were mixed with 15 µl of component A. At t=0 min 15 µl component B was added and the solution was throroughly mixed. After 1 min 30 µl of component C was added, the solution mixed, the injector loop filled, and at t=2 min the loop content injected.

Component B was stable for at least two months when every two days 30 µl mercaptoethanol was added and the reagent components were stored at 4 ^{o}C.

Eluents

All of the eluent components contained 0.1% of Triton X-100. The applied eluents were prepared by mixing 0.1 M sodium-acetate (pH 6.9) and the appropriate organic modifiers by adding volume to volume, and degassing by sonification.

The elution gradient was linear, increasing at a rate of 3% methanol/min.

Chemicals

Double-distilled water made in an all-glass apparatus was used. All other chemicals were of the highest analytical grade available and purchased from Reanal (Budapest, Hungary).

Biological sample preparation

Isolated rat liver hepatocytes were prepared by the two-step collagenase perfusion technique of Seglen /18/. 400 µl samples of cell suspension were incubated in rapidly shaking

centrifuge tubes at 37 °C in nutrient-free medium buffered by HEPES, TES and Tricine.

To terminate incubation the 400 µl cell suspension samples were cooled to 0 °C and centrifuged for 3 min with 1400 rpm. The supernatant of this first centrifugation was used for the extracellular samples and the pellet for the intracellular samples. 75 µl of 10% perchloric acid (PCA) was added to 300 µl of the supernatant. The second centrifugation (5 min, 4000 rpm) resulted in a deproteinized supernatant of which 30 µl was derivatized with OPA. 20 µl of the derivatized product was injected into the HPLC for monitoring extracellular amino acids.

The pellet was treated with 25 µl 10% PCA and diluted with 2% PCA to 375 µl. After a second centrifugation (5 min, 4000 rpm) 30 µl of the deproteinized supernatant was derivatized with OPA. 20 µl of the derivatized product was injected into the HPLC for monitoring intracellular amino acids.

RESULTS

We achieved a good separation on a short HPLC column. It was mostly due to the use of Triton X-100 in the eluent. This non-ionic surfactant increased the resolution of amino acid-OPA derivatives without any loss in column efficiency. In experiments where anionic or cationic surfactants were used, both hydrophobic and ion-exchange equilibria could appear /19/. The most likely consequence of using Triton X-100 could be that secondary equilibria /20/ of the solute molecules are formed as a result of distribution of Triton X-100 between the stationary phase and the eluent on the one hand and between the micelles in the mobile phase and the eluent on the other hand.

We found increased resolution of amino acid dervatives when Triton X-100 was present in the eluent. After the optimization of the eluent composition and gradient we obtained the resolution of amino acid derivatives as illustrated in Fig. 3. In addition to good resolution, a further advantage is the separation of the Gly-, and Thr-OPA adduct. We obtained similar results after the use of tetrahydrofuran (THF) with a combined

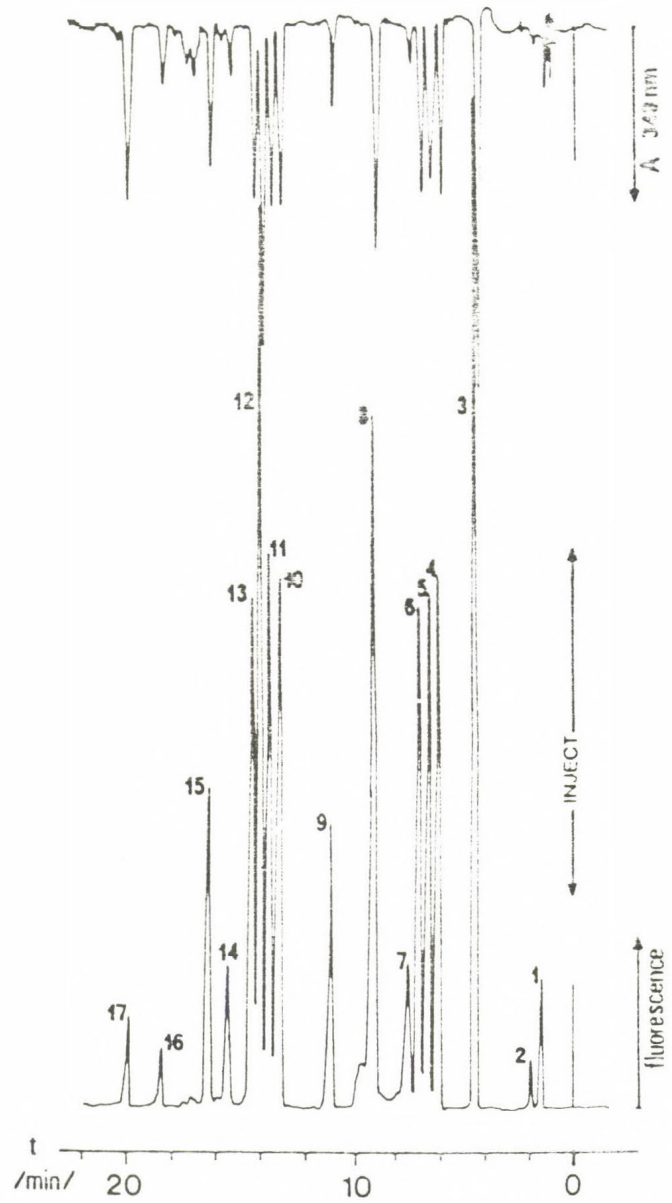

Fig. 3. Separation of a standard amino acid mixture on a 150 x 4.0 mm I.D. SAS-Hypersil column.
Eluent: linear gradient between 5:95:0.1 and 80:20:0.1 (v/v) of methanol: 0.1 M Na-acetate buffer: Triton X-100. Flow rate: 1.55 ml/min. Upper trace: UV absorbance at 340 nm, lower trace: fluorescence (exc.: 340 nm, em.: 425 nm). Each peak represents 1 nmol of amino acid. Peaks: 1: Asp, 2: Glu, 3: Ser, 4: Gln, 5: Gly, 6: Thr, 7: His, 8: Ala, 9: Tyr, 10: Met, 11: Val, 12: Phe, 13: Trp, 14: Ileu, 15: Leu, 16: Orn, 17: Lys

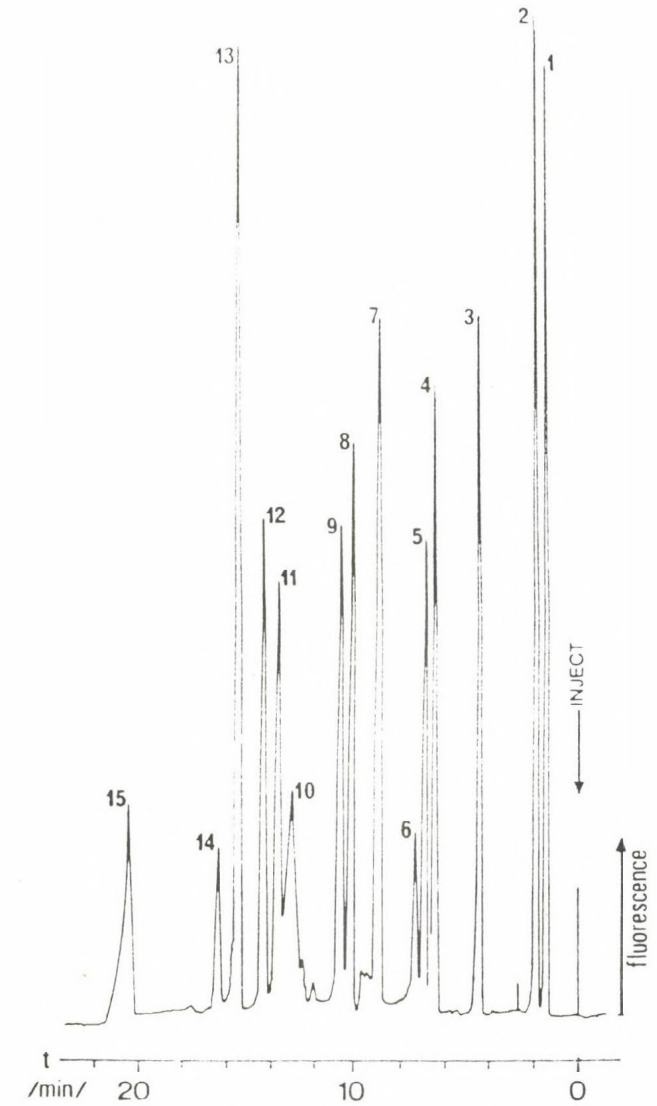

Fig. 4. Amino acids of a peptide hydrolysate.
Column: 150 x 4.0 mm I.D., SAS-Hypersil. Eluent: linear gradient between 5:95:0.1 and 80:20:0.1 (v/v) of methanol: 0.1 M Na-acetate buffer: Triton X-100. Flow rate: 1.55 ml/min. Detection: fluorescence (exc.: 340 nm, em.: 425 nm.)
Peaks: 1: Asp, 2: Glu, 3: Ser, 4: Gly, 5: Thr, 6: His 7: Ala, 8: Arg, 9: Tyr, 10: NH_3, 11: Val, 12: Phe, 13: Ileu, 14: Leu, 15: Lys

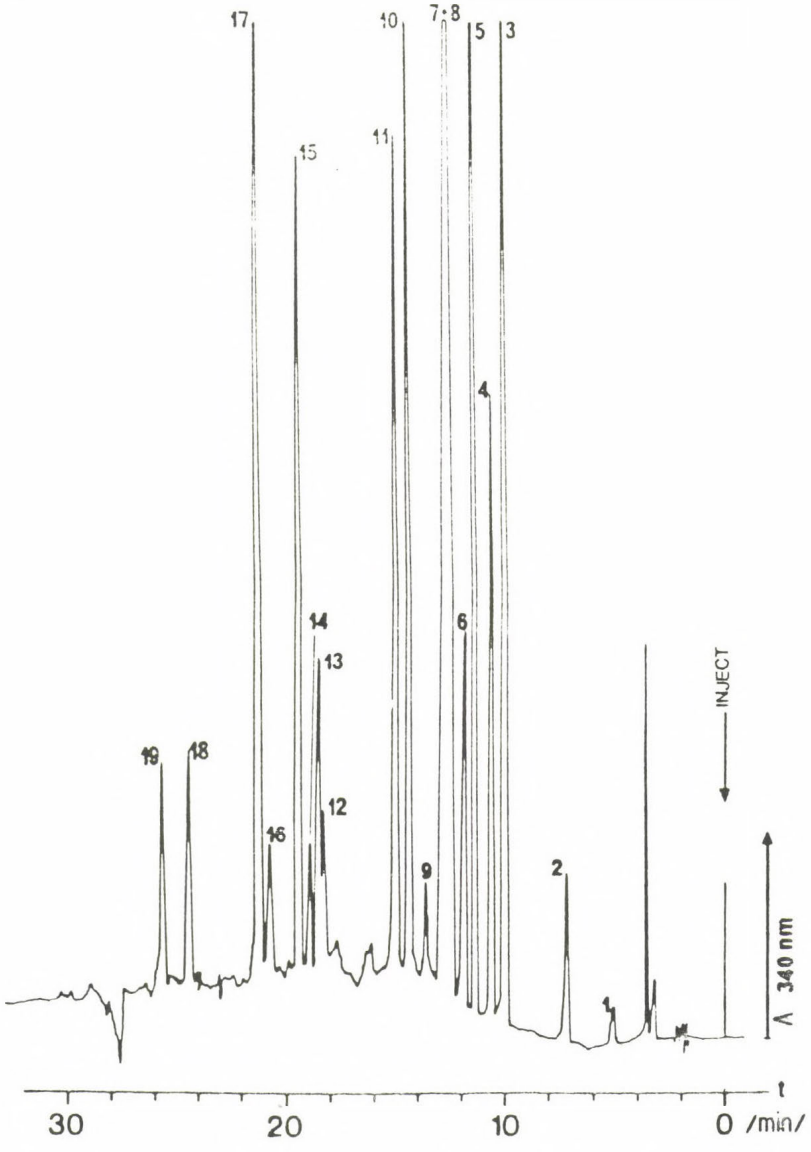

Fig. 5. Analysis of an amino acid mixture on a 250 x 4.0 mm I.D. Chromsil C_{18} column. Eluent: linear gradient between 5:95:0.1 and 80:20:0.1 (v/v) of methanol: 0.1 M Na-acetate buffer: Triton X-100. Flow rate: 1.45 ml/min. Detection: UV at 340 nm. Peaks: 1: 0.15 nmol Asp, 2: 0.45 nmol Glu, 3: 7.5 nmol Asn, 4: 1.5 nmol Ser, 5: 7.5 nmol Gln, 6: 1.5 nmol His, 7: 1.5 nmol Thr, 8: 3.0 nmol Gly, 9: 1.5 nmol His, 10: 3.0 nmol Ala, 11: 3.0 nmol Tyr, 12: 0.45 nmol Met, 13: 1.5 nmol Val 14: 0.45 nmol Trp, 15: 3.0 nmol Phe, 16: 0.9 nmol Ileu, 17: 3.7 nmol Leu, 18: 0.45 nmol Orn, 19: 0.45 nmol Lys

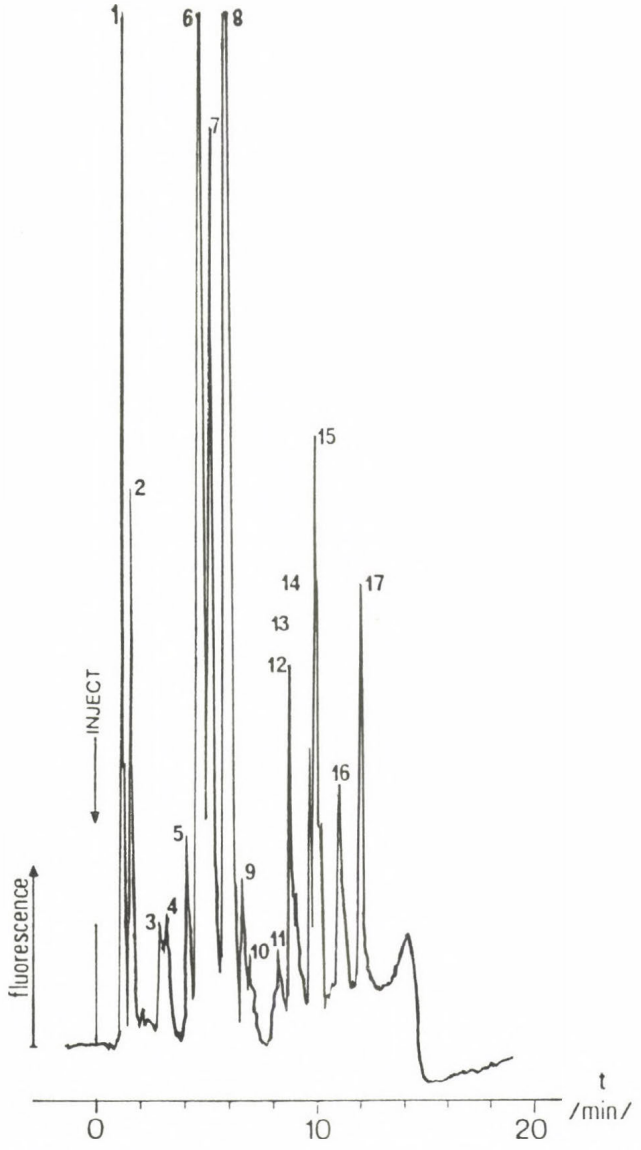

Fig. 6. Amino acid pattern of a sample from isolated rat liver cells. The perchloric acid precipitate of the cells was stored at room temperature for 24 h before chromatography. Column: 250 x 4.0 mm I.D., Chromsil C_{18}. Flow rate 1.45 ml/min, fluorescence detector (exc.: 340 nm, em.: 425 nm). The eluent was the same as in the case of Fig. 5. except that the gradient components contained 1% of tetrahydrofuran. Peaks: 1: Asp, 2: Glu, 3: Asn, 4: Ser, 5: Gln, 6: Gly, 7: Thr, 8: Ala + unknown, 9: unknown, 10: Tyr, 11: Met, 12: Phe, 13: Trp, 14: Ileu, 15: Leu, 16: Orn, 17: Lys

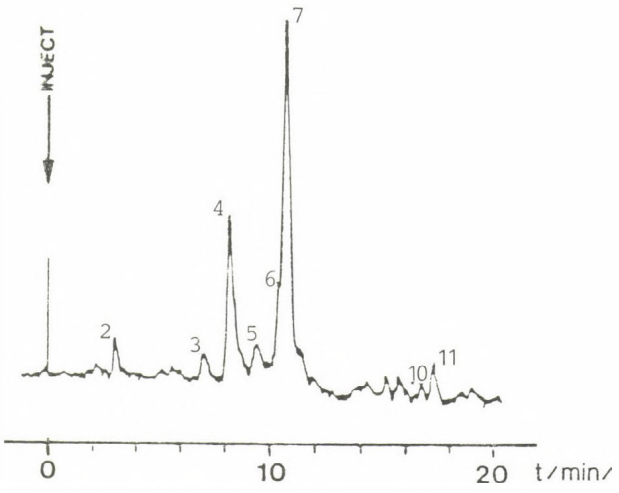

Fig. 7. Amino acids in incubation medium of isolated hepatocytes after 120 min. Column: 150 x 4.0 mm I.D., SAS-Hypersil. Eluent: linear gradient of methanol: 0.1 M Na-acetate buffer: Triton X-100 from 4:96:0.1 to 80:20:0.1. Flow rate 1.32 ml/min. Peaks: 1: Asp, 2: Glu, 3: Gln, 4: Thr, 5: His, 6: Ala, 7: Unknown, 8: Met, 9: Val, 10: Ileu, 11: Leu, 12: Orn, 13: Lys

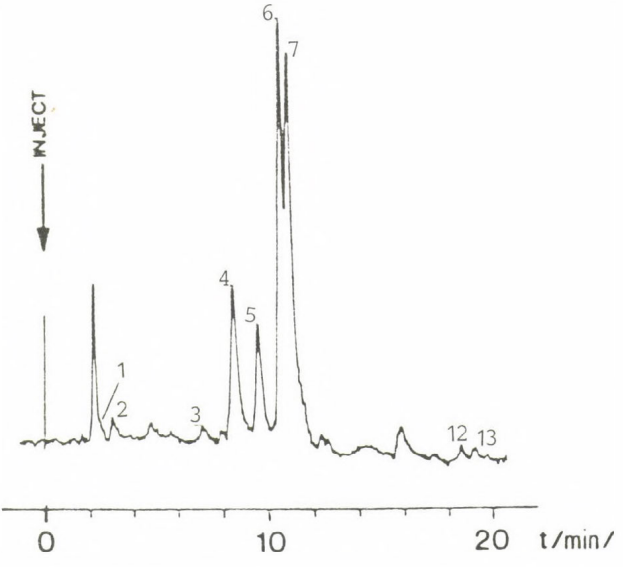

Fig. 8. Amino acid pattern of isolated rat liver cells after 120 min incubation. Conditions and peaks as in Fig. 7.

gradient profile in the case of C_{18} stationary phase (Figs 5. and 6.).

Subsequently we applied our system to the analysis of peptide hydrolyzates (Fig. 4.). To prepare the sample 100 picomole peptide was hydrolized in acido which was followed by lyophilization and redissolution in 100 µl of the eluent. Derivatization was carried out from a 30 µl aliqout.

Figure 5 shows the result of the application of our eluent system on a C_{18} stationary phase /21/. It is obvious that the separation is as good as on the C_1 packing material except for the Gly and Thr adducts which coeluted. In this case the addition of Triton X-100 to the eluent did not effect retention, but the tailing of the peak was suppressed.

Samples deriving from the isolated rat liver cell system were analyzed for extracellular and intracellular amino acid composition. Extracellular amino acids (Fig. 7.) can only originate from the isolated liver cells because the incubation medium originally does not contain amino acids. The chromatograms of intracellular (Fig. 8.) and extracellular samples are qualitatively and quantitatively very different from each other. As the intracellular volume has been very much diluted during sample processing the actual amount of amino acids within the cells is about ten times higher than that seen in Fig. 8.

Both the extracellular, and intracellular samples contain high amounts of Gly and His (peaks 4 and 5). The extracellular samples are also rich in Ileu and Leu (Fig. 7.; peaks 10 and 11), while the intracellular samples in Ala, Orn and Lys (Fig. 8.; peaks 6, 12 and 13).

Extra-, and intracellular samples of 120 min incubation (Fig. 7., 8.) are very similar to those of 30 min incubation (data are not shown here). This means that the partition of amino acids between the cells and the medium is maintained constant by the transport systems during this period.

SUMMARY

Amino acid analysis is an important but time-consuming step in many biological and biochemical investigations. Application of RP-HPLC decreases the analysis time to lass than 40 min. Using the OPA-mercaptoethanol pre-column derivatization procedure the sensitivity allows quantitative amino acid determination at the picomolar level.

This work presents a rapid and sensitive method to separate and quantitate 18 biogenic amino acids. The HPLC of amino acid-OPA derivatives has been carried out on a slurry-packed 150 x 4.0 mm I.D. column using SAS-Hypersil packing material. The enhanced performance and sensitivity has been achieved by a gradient elution system, containing Triton X-100 over the critical micelle concentration and by the optimization of the organic modifier in the eluent. The separation is performed by a routine HPLC equipment and detection of amino acid derivatives is carried out by a tandem UV and fluorescent monitoring system.

The investigated biological samples derive from the isolated rat liver cell system which is widely used for studying cellular metabolism.

ACKNOWLEDGEMENTS

We wish to thank Erika Váradi, Katalin Lőrincz and Zsolt Pálfia for excellent technical assistance.

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COMPARATIVE STUDIES ON THE FREE AMINO ACIDS IN ALFALFA ROOTS, ROOT NODULES AND *RHIZOBIUM* BACTERIA

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ABSTRACT

Free amino acids (FAAs) in the alfalfa roots, root nodules and rhizobia (R. meliloti) were studied by one-dimensional TLC and by an amino acid analyzer. Altogether 16 FAAs were identified. They occur in all studied objects, but their quantitative relationships are different according to the organ and to the host/symbiont combination. Concentration of FAAs is especially high in roots and nodules of plants infected with Nod^+Fix^+ Rhizobium strains. Opine-like compounds could not be detected by the applied methods, however, level of histidine and arginine is lower in the nodules than in the roots, and this may be due to their partial utilization for opine synthesis.

INTRODUCTION

When infected with Rhizobium, leguminous plants develop small, tumor-like outgrowths on their roots, the root nodules, in which fixation of soil dinitrogen takes place via the bacterial nitrogenase. Host specificity, formation and function of the root nodules are determined mainly by the bacterial genes /1/, however, genes of the host plant are also involved /2/. Based upon the analogy of the dicotyledoneous plant cells transformed by the Ti-plasmid of Agrobacterium tumefaciens into tumor cells producing strain-specific amino acid derivatives, the so-called opines (octopine, nopaline etc.), it was assumed

that opine-like compounds can be synthesized and occur also in the root nodules. Therefore, free amino acids in the uninfected roots, root nodules of alfalfa (Medicago sativa L.) and rhizobia (R. meliloti) were studied. Free amino acids of octopine- and nopaline-type tumors and Agrobacterium tumefaciens served for comparison. In the first approach, no attempts were made to detect opines using selective reagents.

MATERIALS AND METHODS

Alfalfa roots, separated root nodules obtained from plants inoculated with effective (Nod^+Fix^+) and ineffective (Nod^+Fix^-) strains of Rhizobium meliloti, rhizobia, Agrobacterium tumefaciens and both octopine- and nopaline-type tumors of Nicotiana plumbaginifolia were fixed and extracted with 80 per cent ethanol. The extracts were cleared by centrifugation and the supernatants were evaporated to dryness under vacuum. The residues were taken up with 80 per cent ethanol, filtered and chromatographed.

Aliquots of the extracts (equivalent with 700 to 900 g dry matter) containing the free amino acids (FAAs) and appropriate standards were spotted on Fixion 50x8 (CHINOIN) ion-exchange sheets and run with a sodium citrate - sodium chloride solvent (Na^+ = 1.5M, pH 6.0, /3/), and alternatively on MN Avicel or MN 300 cellulose layers (250 µm), using repeated development with n-butanol - acetic acid - water 4:1:1 as solvent. Visualization of the spots was performed with a ninhydrin reagent /4/. Some samples were also analyzed on an AMINOCHROM OE-914 (Labor MIM, Budapest, Hungary) automatic amino acid analyzer. Two-dimensional and combined one/two dimensional techniques did not offer any advantage over the applied one-dimensional TLC methods. Ninhydrin-sensitivity of octopine and nopaline was inferior and their Sakaguchi-reaction was also poor.

RESULTS

By means of co-chromatography and color reaction (glycine) the following FAAs could be identified in the samples: leucine, phenylalanine, valine, alanine, glutamic acid, serine, arginine, ornithine, lysine (Figs 1 and 2). Aspartic acid, isoleucine, threonine, proline, tyrosine and histidine were additionally identified in amino acid analyzer (Fig. 3). These amino acids commonly occur in the uninfected and infected roots, root nodules and free rhizobia, however, with different quantitative distribution.

The pattern of FAAs in nodules and uninfected roots is similar, but the nodules are richer in FAAs. Rhizobia and root nodules correspond more closely in their FAA pattern than the nodules and the roots (Figs 1 and 2).

Concentration of FAAs is generally higher in roots and root nodules of alfalfa plants infected by the effective Rhizobium strains as compared to that in the corresponding organs of plants infected with ineffective strains. Especially the amount of glutamic acid, tyrosine, threonine, glycine, aspartic acid, valine and isoleucine increases upon infection in the roots and in the root nodules, but the concentration of some other amino acids also tends to be higher (Fig. 4). Histidine and arginine are exceptions in this respect, since their quantity is lower in the infected roots and in the nodules. Level of proline does not change significantly.

Composition of the FAA pool in R. meliloti and A. tumefaciens is relatively similar, and some FAAs (e.g. leucine, valine, phenylalanine, alanine, glutamic acid, glycine) are certainly common in both the root nodules and the octopine- and nopaline-type tumors. Nevertheless, the FAAs and other ninhydrin-positive compounds of the tumors seem to be more numerous (Fig. 5).

Using the applied separation and detection methods, presence of opine-like compounds in the root nodules could not be showed. But some ninhydrin-positive spots on the chromatograms remained unidentified (for example the strong blue spots, see on the chromatogram depicted by Fig. 2). The low R_f-value com-

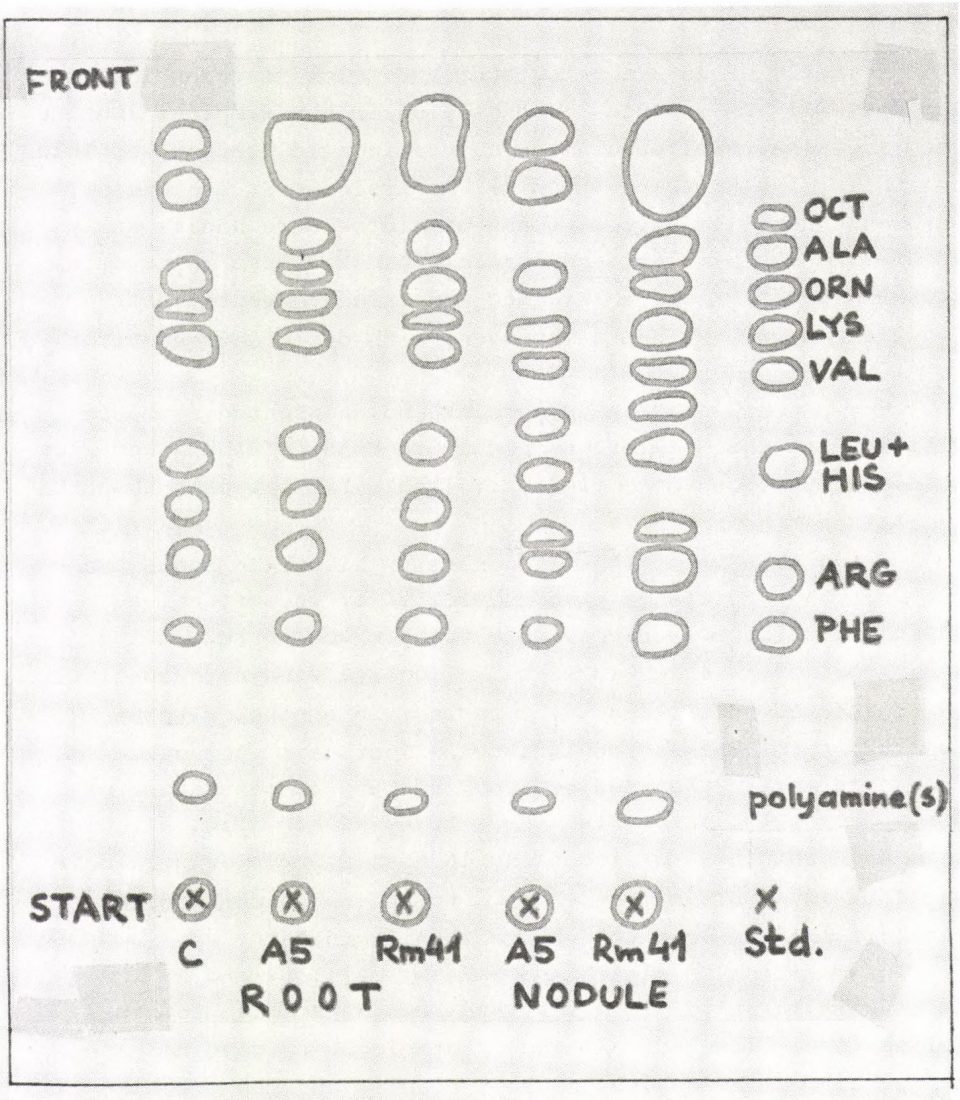

Fig. 1. FAAs from the roots and root nodules of alfalfa plants infected with effective (Rm 41) and ineffective (A5) Rhizobium strains as separated on Fixion 50x8 ion-exchange sheets. C: uninfected roots (controls)

pounds visible on the Fixion sheets (Fig. 1) are probably polyamines.

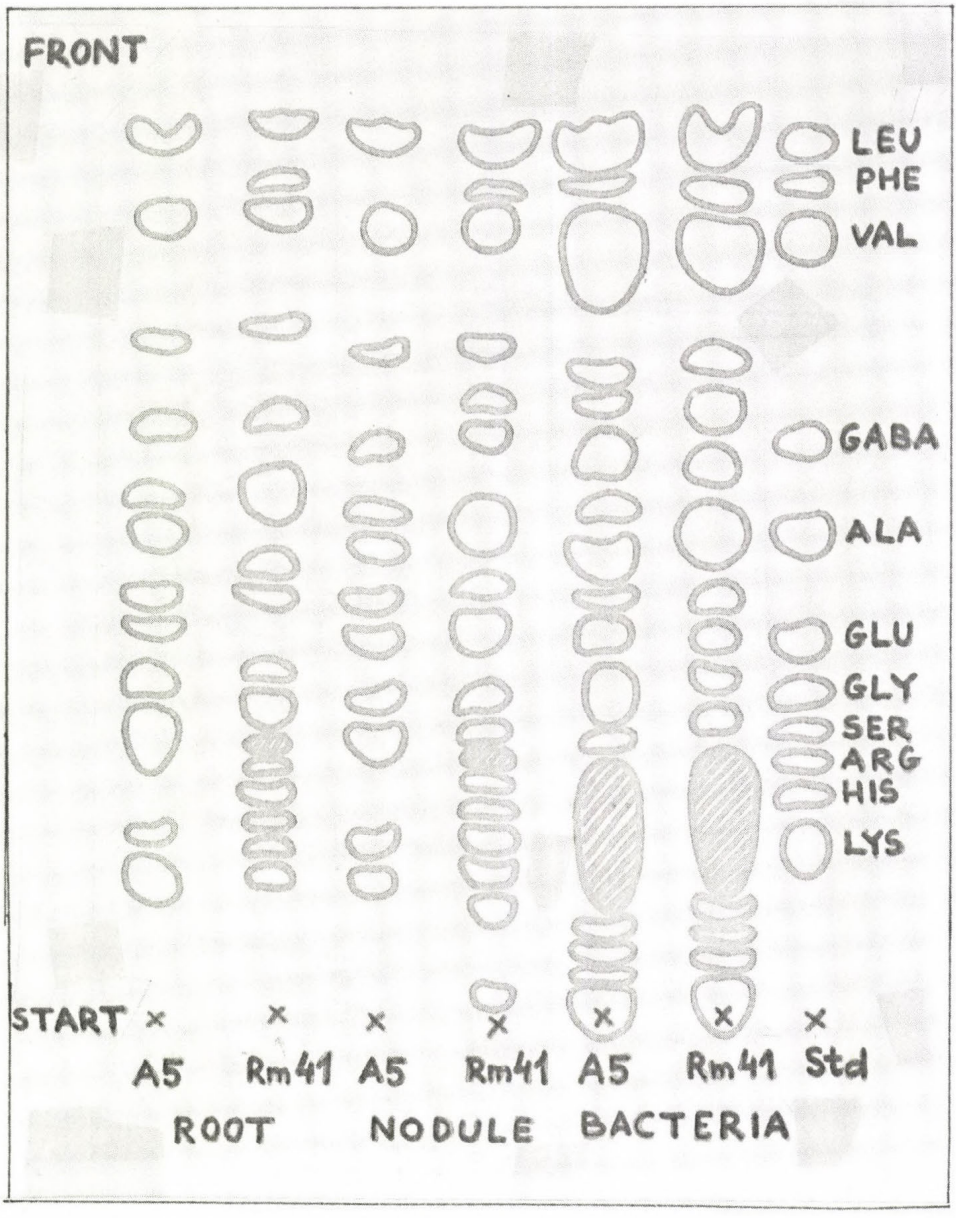

Fig. 2. FAAs from the roots and root nodules of alfalfa plants infected with effective (Rm 41) and ineffective (A5) Rhizobium strains and from the free bacteria of these trains as separated on MN 300 cellulose thin-layer by repeated development

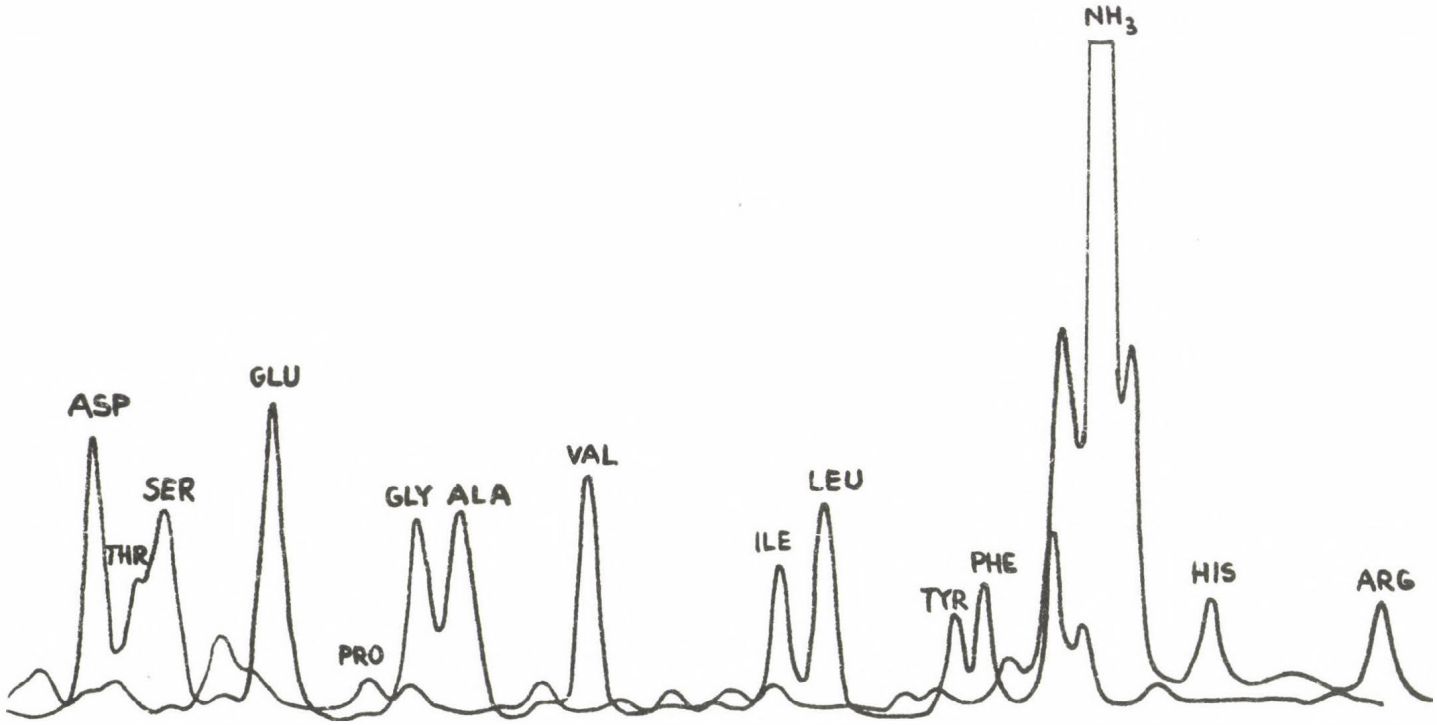

Fig. 3. Automatic amino acid analyzer record of FAAs separated from the extract of ineffective nodules

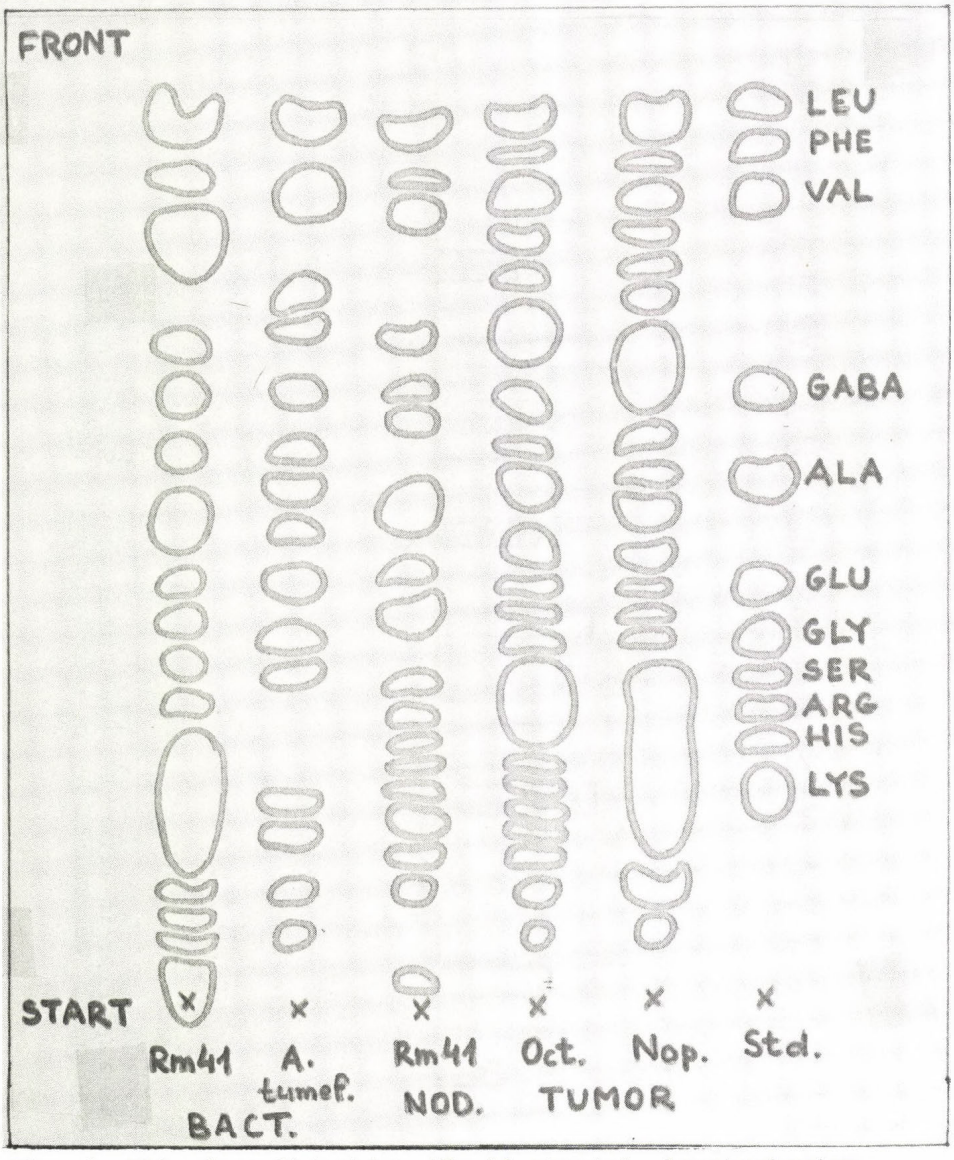

Fig. 4. FAAs from Rhizobium (Rm 41 strain), Agrobacterium tumefaciens, effective nodules and octopine- and nopaline-type tumors as separated on MN 300 cellulose thin-layer by repeated development

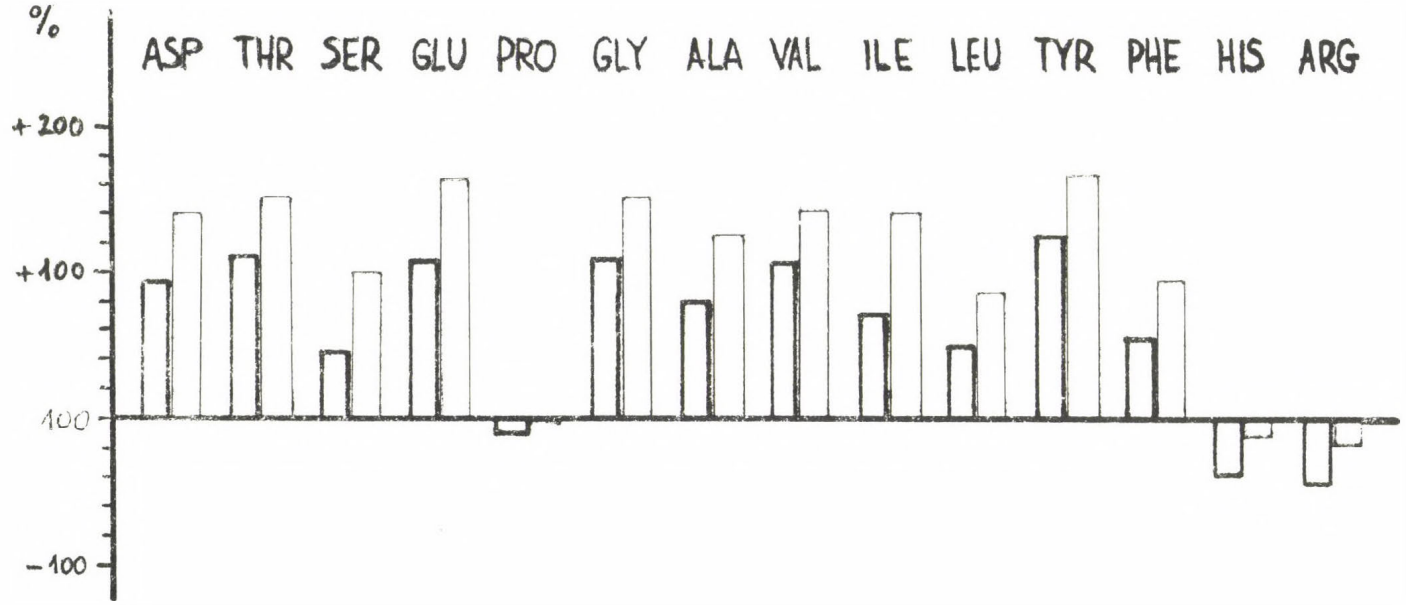

Fig. 5. Per cent change of FAAs in effective □ and ineffective ▨ root nodules as compared to the uninfected roots, determined by an automatic amino acid analyzer

DISCUSSION

It is not surprising that roots and root nodules of alfalfa infected by a Nod^+Fix^+ Rhizobium strain (like Rm 41) contain a higher amount of FAAs, but the stimulation of FAA synthesis by ineffective (Nod^+Fix^-) strains (like A5) is unexpected, and can be explained probably by a wounding effect or a hormonal stimulus exerted indirectly by the symbiont organism. Surplus of FAAs found in roots and root nodules of effective combinations may be then produced to the account of N_2-fixation.

From the "overproduced" FAAs, glutamic acid may have a special importance. According to the model of symbiotic N_2-fixation developed by Shanmugam et. al. /5/, glutamate can play a key role in the regulation of fixed N-export from the nodules. However, increased level of glutamic acid in the ineffective nodules cannot be explained by this way.

Differential presence of the not identified "blue compound" of the effective and ineffective rhizobia both in the root nodules and the nodule-free roots can open interesting possibilities if confirmed by further experiments. Namely, this compound seems to be transported from the bacteria and the nodules to the roots in the effective symbioses only (Fig. 2). Thus, it could serve as a simple marker for N_2-fixing host/Rhizobium combinations.

Although opine-like derivatives could not be detected in the root nodules formed after Rhizobium-infection on alfalfa roots, their occurrence cannot be excluded. To obtain a definite answer, more concentrated extracts and more sensitive and specific reagents (like phenanthrence chinone) should be used. However, decrease of histidine and arginine content in the nodules of some combinations (Fig. 4) can be in connection with their partial utilization for opine synthesis.

ACKNOWLEDGEMENT

The authors are indebted to M. Czakó for the octopine- and nopaline-tumors. This work was supported by OMFB-FPI under contract No. 268/82 and 376-2/83.

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HPLC OF PEPTIDES ON OCTADECYLSILICA AND UNMODIFIED SILICA

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SUMMARY

The relationship between the capacity factors of peptides and the acetonitrile concentration in the mobile phase has been studied. It is shown that the plots of log k' vs. the logarithm of acetonitrile molarity are parabolic both for Zorbax ODS octadecylsilica and unmodified Silasorb 600 silica. At high acetonitrile concentrations the absolute retentions on the two packings are similar. The possible mechanisms of retention and the applications of unusual modes of peptide HPLC are briefly discussed.

INTRODUCTION

Reversed-phase HPLC has become one of the main techniques in the investigations of peptides during the last decade. The theory shows /1/ that in the reversed-phase mode the retention of solutes depends mainly on the solvophobic effect, and the dispersion interaction between the solute molecules and the alkyl ligates of the stationary pahase is much less important. It has been demonstrated /2/ that the hydrophobic interaction is the basic mechanism governing the chromatography of peptides. Retention in the reversed-phase mode usually decreases when the concentration of organic modifier in the mobile phase increases. Such behaviour has also been reported for peptides in several papers /2-4/.

The relationship between retention and composition of the mobile phase can be described by the following expression /5/:

$$\log k' = b + p \log C \qquad (1)$$

where k' is the capacity factor, and C is the acetonitrile molarity. In the reversed-phase mode the sign of p is negative.

On the other hand, some researchers /6-8/ have shown that the shape of the log k' vs. log C plots for some peptides does not correspond to Eq. 1, being not linear, but parabolic. At low concentrations of the organic modifier the retention of peptides decreases when C is increased, but at concentrations higher than 40-70% the opposite is true.

One of the possible explanations of the observed phenomena is a dual retention mechanism involving silanophilic retention, which dominates at high concentrations of the organic modifier of the mobile phase.

EXPERIMENTAL

The capacity factors (k') of peptides were calculated according to the usual expression:

$$k' = \frac{t_R - t_o}{t_o} \qquad (2)$$

where t_R stands for the retention time of the solute studied, and t_o is the time of emergence of an unretained solute. Sodium nitrate was used as the unretained marker.

The Du Pont models 830 and 850 HPLC instruments were used in our work.

The two columns used were 250 x 4.6 mm Zorbax ODS (octadecylsilica) and 150 x 4.6 mm Silasorb 600 (unmodified silica). The particle size of both packings was 5 μm. The mobile phases consisted of acetonitrile (pure) and 0.1 M phosphate (pH 2.5) or 0.1 M acetate (pH 5) buffer. The mobile phase flow was

1.5 ml/min. The UV-detection of the peptides was performed at 220 or 254 nm.

The studied synthetic peptides had the following amino acid composition:

I	Ala-1, Ile-1, Lys-1, Phe-1, Pro-1, Tyr-1, Val-1
II	Arg-1, His-1, Ile-1, Lys-1, Phe-1, Pro-1, Tyr-1, Val-1
III	Glu-1, His-1, Lys-1, Pro-1, Ser-1, Tyr-1,
IV	Arg-1, Gly-2, Lys-1, Phe-2, Pro-3
V	Arg-2, Asn-1, His-1, Phe-1, Pro-1, Tyr-1, Val-1
VI	Arg-1, Asn-1, His-1, Phe-1, Pro-1, Tyr-1, Val-2
VII	Arg-1, Lys-1, Gly-1, Pro-1, Trp-1
VIII	Gly-1, D-Orn-1, Phe-1, Tyr-1
IX	Arg-1, Gly-1, Lys-1, Pro-1

RESULTS AND DISCUSSION

The retention data of the compounds studied are given in Table 1. Fig. 1 shows the parabolic relationship between the capacity factors and the concentration of acetonitrile. Such relationship is unusual for hydrophobic chromatography and indicates a more complex retention mechanism. At high concentrations of acetonitrile or methanol the adsorption on the residual silanol groups of the silica matrix increases, which is supposed to result in stronger retention /6, 7/.

Adsorption on residual silanol groups is not the only possible mechanism. For example E. Papp and Gy. Vigh /9/ have shown that the nature of "silanophilic" retention of aromatic amines is ion exchange, and the contribution of such mechanism is large over the entire organic solvent concentration rage. We believe that the data available now are not sufficient to rigorously identify the retention mechanism of peptides on alkylsilicas at a high percentage of the organic modifier.

The theoretical analysis and precise measurements of k' of various compounds made by Schoenmakers et al. /10/ have shown that the actual behaviour in RP HPLC does not strictly follow Eq. 1 and that a second degree term must be introduced:

Table 1. The values of log k′ on Zorbax ODS
(P - phosphate buffer; A - acetate buffer)

Compound	Buffer	Acetonitrile concentration, vol. %						
		15%	20%	30%	45%	50%	60%	70%
I	P		0.73	-0.15	-0.57	-0.48	-0.22	0.07
	A			0.37	-0.03	-0.18	-0.29	
II	P		0.58	-0.28	-0.52	-0.40	-0.07	0.37
	A			0.46	0.25	0.16	0.33	0.49
III	P	0.16	-0.25	-0.68	-0.70	-0.30	-0.15	0.29
	A	0.33	-0.13		-0.25	-0.11	0	0.44
IV	P		1.13	0.25	-0.17	-0.11	0.11	0.47
	A			0.68	0.54	0.50	0.69	1.01
V	P	0.57	-0.06	-0.70	-0.70	-0.43	0.06	0.73
	A		0.58		0.09	0.24	0.50	0.98
VI	P	0.97	0.32	0.42	-0.58	-0.48	-0.10	0.40
	A	1.19	0.46		-0.19		0.23	0.50
VII	P		0.85	-0.01	-0.48	-0.43	-0.24	0.04
	A		0.69		-0.01	-0.04	0.40	0.45
VIII	P	-0.31	-0.55	-0.74		-0.57	-0.30	0.12
	A	-0.13	-0.30		-0.18	-0.14	0	0.32
IX	P				-0.70	-0.33	0.20	0.92

$$\log k' = A + B\rho + C\rho^2 \qquad (3)$$

where ρ is the volume fraction of the organic modifier.

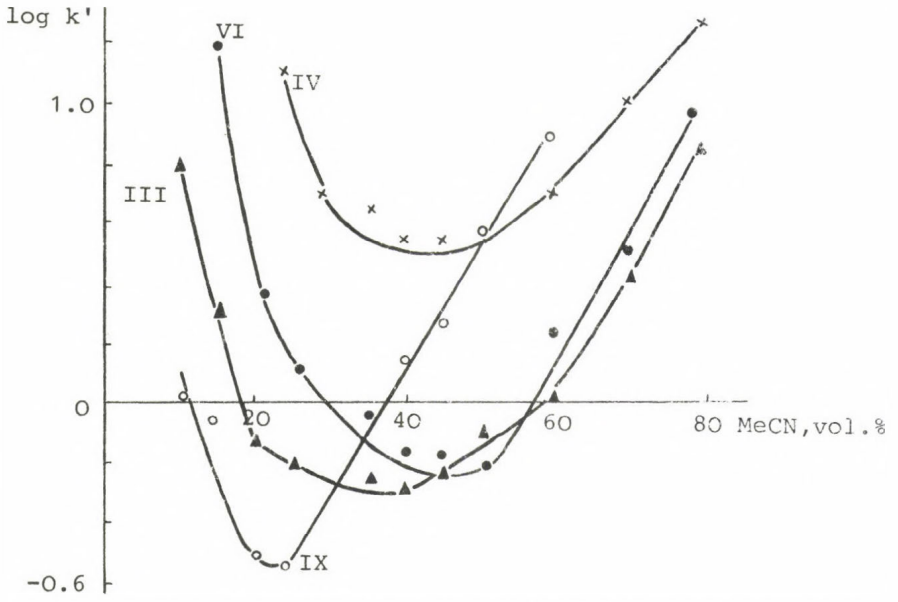

Fig. 1. The relationship between acetonitrile concentration and the retention of peptides. Zorbax ODS, phosphate buffer

Nevertheless, the curvature observed by Schoenmakers is small and the second degree term in Eq. 3 gives only a minor improvement in the description of data.

The situation is quite different with the peptides studied here. For this class of compounds studied over a wide C range, Eq. 1 cannot be used and the application of some quadratic equation is necessary to describe their chromatographic behaviour. The reason for such behaviour can be the dual retention mechanism, coexistence of two retention modes in which the opposite and non-linear change of retention occurs when the concentration of acetonitrile is changed.

The retention values of these peptides can be described by the following equation:

$$\log k' = a_o + a_1 \log C + a_2 \log^2 C \qquad (4)$$

which is a modification of Schoenmakers' relationship. The agreement between eq. 4 and the experimental values is demonstrated by the correlation data given in Table 2.

It is interesting to compare the elution sequence of peptides at high and low concentrations of acetonitrile. It is known from the literature /7/ that at high acetonitrile concentrations the elution order becomes reversed and is opposite to the order observed at low concentrations. If the elution order is not changed, the retention values at high and low C should be correlated and the slope of the correlation curve should be positive. If the opposite is true the slope will be negative. The data shown is Fig. 2 indicate that the relationship is more complicated and that only trends can be discussed. In the presence of acetate buffer at high acetonitrile concentrations the "reversed-phase" elution order is maintained. However, it can be seen that the selectivity of separation of pairs of peptides is altered. When phosphate buffer is used as a mobile phase constituent, in many (but not in all) cases the elution order was reversed.

The observed behaviour of peptides and the possible role of unmodified silica sites have led us to a series of experiments where bare silica was used as the stationary phase. The mobile phases were identical to those used in the first part of the work. The obtained retention values are given in Table 3. It is apparent that at high C the capacity factors do not differ much from the values observed on Zorbax ODS. This fact shows that under the present experimental conditions hydrocarbon ligates of ODS-packing do not play any significant role in retention. The parabolic relationship between retention and C is also characteristic of unmodified silica (Table 4).

The general trends in elution behaviour on the two packings are compared in Fig. 3. It can be seen that at high C the

Table 2. The parameters of Eq. 4. Column Packing-Zorbax ODS

Compound	Buffer	a_0	a_1	a_2	Correlation coefficient
I	P	8.84	-20.4	11.1	0.99
	A	7.14	-13.6	6.3	0.99
II	P	9.14	-22.0	12.5	0.99
	A	9.27	-19.4	10.3	0.96
III	P	3.40	-10.6	6.9	0.97
	A	2.84	- 8.4	5.5	0.97
IV	P	9.62	-21.3	11.6	0.99
	A	9.22	-19.2	10.5	0.99
V	P	7.34	-20.5	12.9	0.98
	A	6.44	-15.3	9.2	0.96
VI	P	7.23	-18.5	11.0	0.98
	A	6.13	-14.8	8.8	0.99
VII	P	9.35	-21.2	11.5	0.99
	A	6.44	-14.6	8.3	0.95
VIII	P	1.75	- 6.8	4.7	0.97
IX	A	1.09	- 4.8	4.3	0.97

behaviour of peptides on Zorbax ODS and Silasorb 600 is almost identical.

At low C, a correlation between two sets of data exists, but the separation selectivity for certain pairs of peptides is different.

The column efficiencies obtained for the peptides were about 2000 and 1000 theoretical plates for ODS and silica columns, respectively. These values are much lower than the efficiency of the columns used against small, non-ionizable molecules. Taking into account that the Silasorb 600 column was shorter than the Zorbax ODS column it may be concluded that the efficiency of the two packings against peptides is similar.

Thus, the results obtained on the two different packings with the mobile phases consisting of two buffers and widely varied acetonitrile concentration formally follow the dual retention mechanism model. At the same time, the retention data

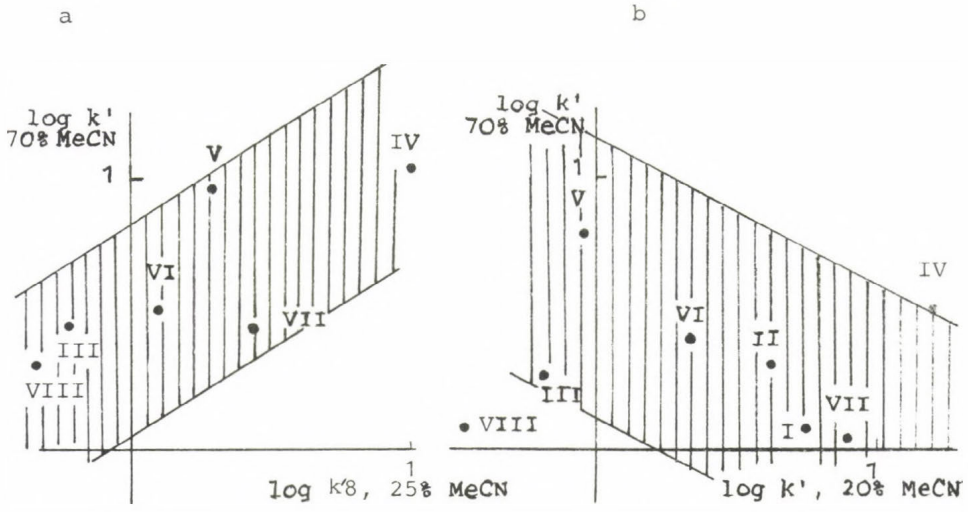

Fig. 2. The trends of retention sequence on Zorbax ODS compared at high and low acetonitrile concentration. a - Acetate buffer, b - phosphate buffer

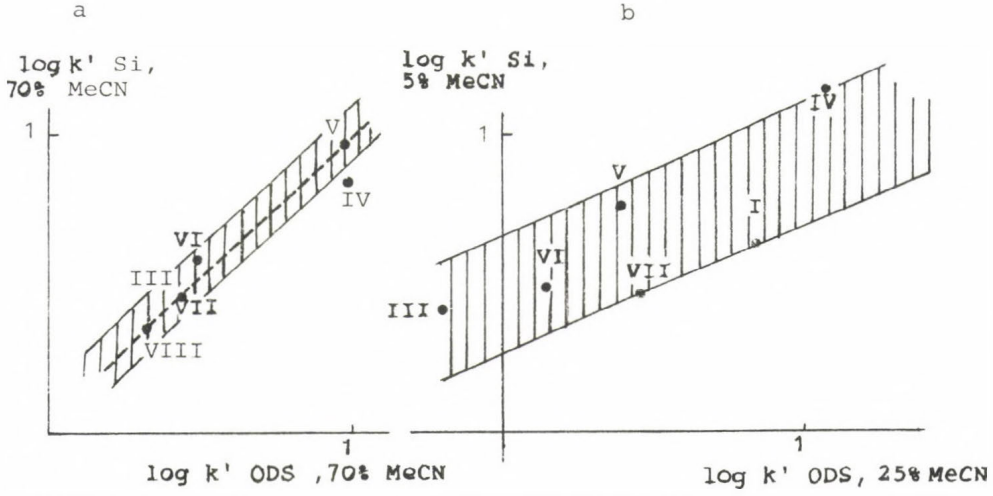

Fig. 3. The comparison of retention on Zorbax ODS and Silasorb 600. a - the right branch of parabola Eq. 4, b - the left branch of the parabola

Table 3. The values of log k' on Silasorb 600. Mobile phase: buffer A + acetonitrile

Compound	MeCN, Vol. %									
	90	80	70	60	50	40	20	10	5	0
I	0.70	0.15	0	-0.13	0.30	-0.52	-0.46	0	0.63	0.90
III	-	0.98	0.48	0.26	0.20	0	0.04	0.18	0.42	0.51
IV	-	0.98	0.82	0.58	0.53	0.38	0.36	0.53	1.18	1.56
V	-	1.62	0.95	0.62	0.50	0.34	0.27	0.26	0.77	1.18
VI	-	1.04	0.56	0.26	0.18	0	-0.10	0	0.48	0.88
VII	-	0.88	0.42	0.15	0.08	0.08	0.11	0.20	0.46	0.81
VIII	1.85	0.73	0.34	0.15	0.08	-0.10	-0.70	-1.0	-1.0	-1.0
IX	-	-	1.56	1.04	0.83	0.42	0.06	0.146	0.27	0.40

Table 4. The Parameters of Eq. 4. Column Packing-Zorbax ODS Buffer - A

Compound	a_o	a_1	a_2	Correlation coefficient
I	0.65	-3.71	2.86	0.94
III	0.46	-2.01	1.87	0.86
IV	1.12	-2.66	2.10	0.98
V	0.77	-2.77	2.70	0.91
VI	0.48	-2.60	2.43	0.93
VII	0.50	-1.80	1.57	0.80
IX	0.31	-1.97	2.59	0.97

on bare silica allow to doubt the significant role of alkyl ligates of the ODS-packing not only at high, but also at low acetonitrile concentrations. The present results cannot be explained satisfactorily either by hydrophobic (solvophobic) sorption or by in exchange, or by adsorption on polar silica sites. Unless the mechanism of such complex behaviour of peptides remains unknown, the discussed phenomena may have wide application and offer the following possibilities in the field of analytical and preparative HPLC of peptides:

- the availability of acceptable k' values on ODS-silicas for highly polar peptides, which are not retained at low C in usual RP separations. High C should be used for this purpose (see compound VIII, Table 2),
- the possibility of variation of separation selectivity on ODS-silica. Higher C with approximately the same elution strength should be used for this purpose (Fig. 2),
- the possibility of modifying the selectivity of separation by the use of bare silica instead of ODS-packing (Fig. 3),
- the possibility of making the preparative separations cheaper by the use of unmodified silica and reduced concentration of organic solvent in the mobile phase.

Fig. 4. shows some examples of such non-traditional modes of HPLC for peptides.

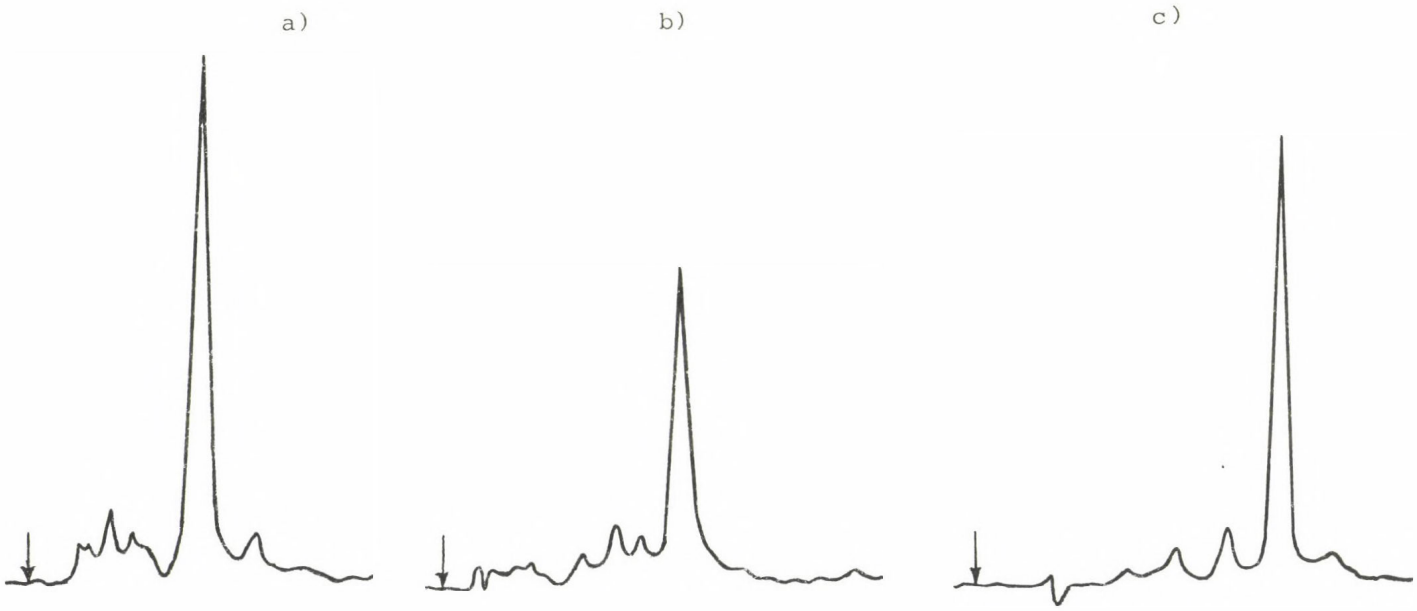

Fig. 4. Non-traditional modes of HPLC of peptides.
a) compound IV, Silasorb 600, buffer A, 20% MeCN
b) compound VI, Silasorb 600, buffer A, 70% MeCN
c) compound VI, Zorbax ODS, buffer A, 70% MeCN

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RAPID QUANTITATIVE N-TERMINAL ANALYSIS OF PEPTIDES BY REVERSED PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Determination of N-terminal amino acids as 2-dimethylamino-naphthalene-5-sulfonyl (DNS)-derivatives is one of the most useful criteria of peptide purity. High sensitivity and simplicity made this method popular in protein chemistry. Many techniques for DNS-amino acid identification including reversed-phase HPLC, the most promising procedure for the separation of these derivatives, are described. However, now it is not easy to utilize all the advantages of HPLC since conventional conditions for dansylation of peptides complicate the subsequent separation of DNS-amino acids. As a rule separations of standard mixtures of DNS-amino acids are reported in the literature, but not of DNS-amino acids obtained by dansylation of peptides followed by hydrolysis of DNS-peptides.

The present investigation was aimed at the development of a procedure for rapid quantitative N-terminal analysis at subnanomole level of peptides consisting of 5-30 amino acid residues, particularly hydrophobic fragments of membrane proteins. This procedure could be of value for purity control of peptides before their sequencing.

EXPERIMENTAL

The procedures for peptide dansylation, hydrolysis of DNS-peptides as well as for the separation of DNS-amino acids have been described earlier /1/.

SEPARATION OF STANDARD MIXTURE OF DNS-AMINO ACIDS

To obtain reproducible chromatographic results for a mixture of many DNS-amino acids it was necessary to find the optimal conditions for the separation process (nature, molarity, and pH of buffer solution, as well as optimum temperature). Such an optimization was described elsewhere /1/.

Fig. 1. shows the separation pattern of the standard mixture containing 23 mono- and di-DNS-derivatives of amino acids (60-100 picomoles each) as well as dansyl acid (DNS-OH) and dansyl amide ($DNS-NH_2$). Complete separation of this mixture takes 26 min, equilibration of the column takes 5 minutes more It can be mentioned that O-DNS-Tyr and ε-DNS-Lys give diffuse peaks and DNS-Asp and DNS-Glu peak areas were half those of equimolar amounts of other derivatives.

DANSYLATION OF PEPTIDES

When dansylation of amino acids and peptides is performed according to the conventional methods the chromatographic pattern considerably differs from that shown in Fig. 1. A large excess of DNS-Cl results in the formation of great amounts of side products which complicate identification of many DNS-amino acids. Therefore, such conditions of dansylation had to be found which would permit the subsequent unequivocal identification of all 23 DNS-amino acids. Dansylation of peptides was carried out in lithium carbonate buffer as recommended by Tapuchi et al. /2/. Five to ten-fold excess of DNS-Cl was found to be sufficient, instead of 100-1000-fold excess used in the conventional procedures.

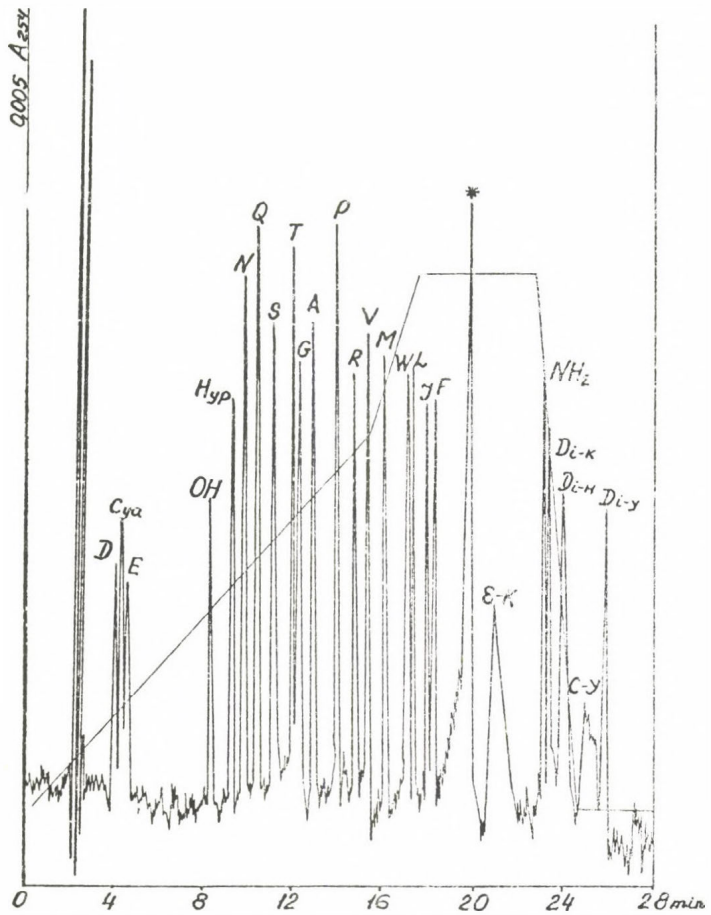

Fig. 1. Reversed-phase HPLC of DNS-amino acids on an Ultrasphere ODS (5 µm) column (250x4.6 mm I.D.), 100 pmol of each derivative, except the di-derivatives (60 pmol of each). Solvent A, 10:90 (v/v), acetonitrile-sodium TFA buffer (25 mM, pH 7.6), solvent B, 70:30 (v/v) acetonitrile-sodium TFA buffer (25 mM, pH 7.6) gradient from 7 to 65% B. Solvent program: 0 min after injection, 7% B, 1 min, 7%, 16 min, 48%, 18 min, 65%, 23 min, 65%, and 25 min, 7%. Detector range, 0.005 a.u. f.s. at 254 nm. Flow-rate 1.2 ml/min, temperature 39°C. One-letter code is used for the amino acids (D8=Asp, E=Glu, N=Asn, Q=Gln, S=Ser, T=Thr, G=Gly, A 9 Ala, P=Pro, R=Arg, V=Val, W=Trp, M=Met, L=Leu, I=Ile, F=Phe) except for the following abbreviations: Cya=cysteic acid, Hyp=hydroxyproline, Di-K=Di-DNS-Lys, Di-H=Di-DNS-His, Di-Y=Di-DNS-Tyr, ε-K=ε-DNS-Lys, O-Y=O-DNS-Tyr, OH=DNS-sulphonic acid, NH_2=DNS-amide. The peak marked with an asterisk corresponds to an unknown compound

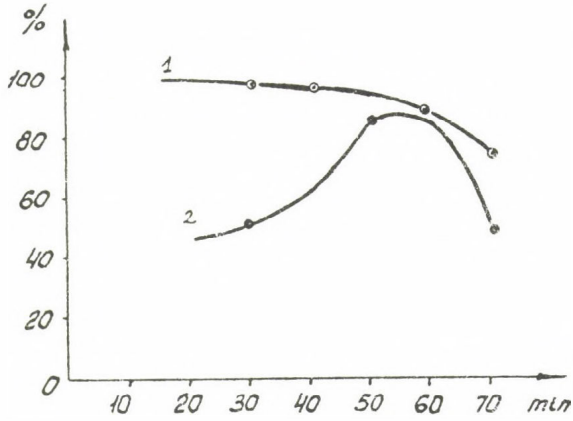

Fig. 2. Time dependence of acid hydrolysis. (HCl/TFA, 2:1, 166°): 1 recovery of DNS-Phe after its hydrolysis, 2 yield of DNS-Phe from dansylated insulin B-chain

We found that running the reaction in a small volume (6 µl) at small excess of the reagent provides complete dansylation and minimum formation of side products. Dansylation of a sample in the constant volume, irrespective of the amount of the peptide increases the reproducibility of the results.

HYDROLYSIS OF DNS-PEPTIDES

The conventional procedures for the hydrolysis of DNS-peptides involve treatment with 5.7 M HCl for 4-12 hours at 105° and result in considerable destruction of the DNS-derivatives of Met, Pro, Gly. They are not suitable for a rapid analysis. In order to accelerate the hydrolysis of DNS-peptides we employed a mixture of 5.7 M HCl and trifluoroacetic acid (TFA) which was earlier suggested for peptide hydrolysis by Tsugita and Scheffer /3/.

Fig. 2 shows the dependence of the yield of DNS-Phe on the duration of hydrolysis of the dansylated insulin B-chain (curve 2) and the kinetics of DNS-Phe destruction (curve 1). It is seen that the decrease in the yield of DNS-Phe under these con-

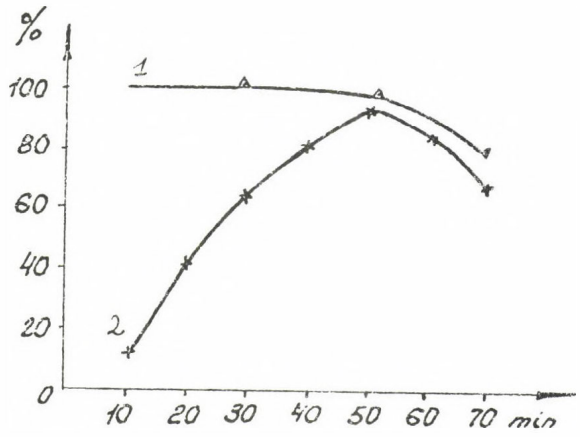

Fig. 3. Time dependence of acid hydrolysis (2:1 HCl/TFA, 166°): 1 recovery of DNS-Ile after its hydrolysis, 2 yield of DNS-Ile from dansylated Ile-Pro-Gly-glu

ditions is due to its destruction. Similar situation is observed in the case of DNS-Ile-pro-Gly-Glu (Fig. 3, curve 2) and DNS-Ile (Fig. 3, curve 1). On the basis of these data we concluded that the optimum time for hydrolysis of DNS-peptides containing up to 30 amino acids using the 2:1 mixture of hydrochloric and trifluoroacetic acids (TFA) is 50 min, at 166°.

The extent of the destruction of some DNS-amino acids under these conditions is shown in table 1.

Hydrolysis of DNS-peptides with the 5.7 M HCl/trifluoroacetic acid mixture allowed not only to shorten the hydrolysis time, as compared with 5.7 M HCl hydrolysis (from 4-12 hours to 50 minutes), but also to obtain high yields for the N-terminal DNS-amino acids (table 2).

We have examined the applicability of the described procedure for determining the N-terminal DNS-amino acids in the case of sequences such as Val-Val, Ile-Val or Ile-Leu. These combinations can be found quite often in hydrophobic fragments of membrane proteins and are poorly hydrolyzable. The splitting of the respective model dipeptides required 100-min hydrolysis (see Fig. 4), the yield of N-terminal DNS-amino acid being

Table 1. Recovery of DNS-amino acids after their hydrolysis (5.7 M 2:1 HCl/trifluoroacetic acid, 166°C, 50 min.)

No.	DNS-amino acid	Yield, %
1	DNS-Gly	83
2	DNS-Ser	75
3	DNS-Thr	80
4	DNS-Ala	91
5	DNS-Val	91
6	DNS-Ile	90
7	DNS-Leu	85
8	DNS-Phe	85
9	DNS-Pro	58
10	DNS-Met	65
11	Di-DNS-Lys	75
12	Di-DNS-Tyr	80
13	Di-DNS-His	60

Table 2. Yield of N-terminal DNS-Phe at acidic hydrolysis of insulin B-chain*

No.	Conditions	Yield, %**	Relative yield***
1	5.7 M HCl, 105°, 18 h	47±6	0.71
2	5.7 M HCl, 105°, 12 h	50±6	0.76
3	5.7 M HCl, 105°, 4 h	66±6	1.00
4	5.7 M HCl + TFA, (2:1) 166°, 50 min.	83±4	1.26

*190 picomoles of insulin B-chain analysed, 160 picomoles injected into HPLC column,
**mean value of 6 determinations,
***yield for No. 3 considered as 1.00.

1.5-2-fold higher than compared with conventional hydrolysis (Table 3).

Thus the use of the suggested procedures for the dansylation of peptides, hydrolysis of DNS-peptides, and separation of

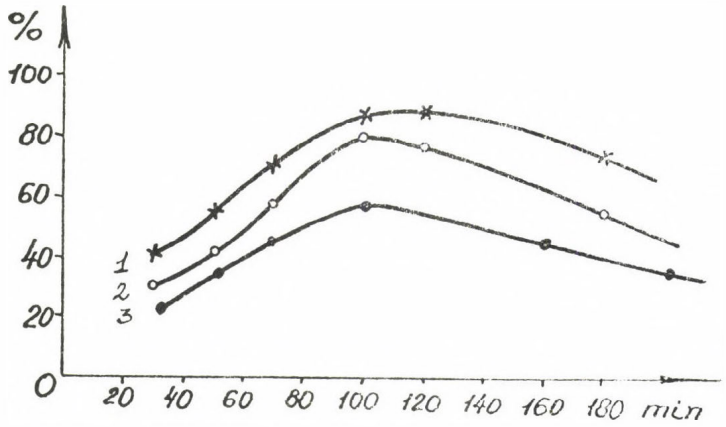

Fig. 4. Time dependence of the yield of N-terminal DNS-amino acids of poorly hydrolyzable DNS-dipeptides: 1 DNS-Val-Val, 2 DNS-Ile-Val, 3 DNS-Ile-Leu

Table 3. Yield of N-terminal DNS-amino acids in the hydrolysis of the dansylated hydrophobic dipeptides

Dipeptide	Yield, %*			Relative yield
	HCl 105° 18h	HCl/TFA, 166° 50 min	HCl/TFA, 166° 100 min	
	I	II	III	(III/I)
Val-Val	60	70	87	1.45
Ile-Val	57	58	80	1.40
Ile-Leu	25	45	57	2.20

*mean value of 6 determinations

DNS-amino acids permits to rapidly and quantitatively perform the N-terminal analysis. The total analysis time is shortened to 2-3 hours with a simultaneous increase of up to 80-85% in the yield of DNS-amino acids. The discussed procedure seems to be useful in the study of the primary structure of proteins that are available in minute amounts. In particular, it seems promising for purity control of membrane protein fragments.

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HPLC PURIFICATION OF TRITIATED PEPTIDE HORMONES

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ABSTRACT

Vasopressin analogues (AVP, dDAVP and dGAVP) and cholecystokinin octapeptide (CCK-8) were tritiated through their diiodotyrosine-containing analogues during catalytic dehalogenation. The labeled peptides were purified on a Sep-Pak C-18 cartridge, followed by reversed-phase high-performance liquid chromatography. This isolation method gave peptides of high purity and high specific radioactivity (16-42 Ci/mmole). The tritiated hormones proved to be biologically active in physiological experiments.

INTRODUCTION

For the investigation of the metabolism and receptor binding of peptide hormones, radiolabeled substances of high specific radioactivity and full biological potency are needed. Although the radioiodinated compounds have extremely high specific activities, these substances cannot be applied in all assays because the biological activity of the iodine labeled peptide usually differs from that of the unlabeled hormone. However, tritium-labeled materials retain all of the features of the native molecules. Catalytic dehalogenation with tritium is a favoured method of producing substances labeled on the Tyr or Phe residues. The isolation of the desired tritiated compounds from the crude reaction mixture by the earlier chromato-

graphic methods is difficult. Although reversed-phase high-performance liquid chromatography (RP-HPLC) is a powerful method for the analysis and separation of peptides, it has not been used extensively to produce highly-purified tritium-labeled peptide hormones.

The present paper reports on the application of RP-HPLC in the preparation of pure, biologically active tritiated vasopressin analogues and cholecystokinin octapeptide (CCK-8) of high specific radioactivity.

MATERIALS AND METHODS

Radioactive materials

The $(3,5\text{-diiodo-Tyr})^2$-containing vasopressin analogues 8-Arg-vasopressin (AVP), 1-deamino-8D-Arg-vasopressin (dDAVP) and desglycinamide-8-Arg-vasopressin (dGAVP) were prepared by the method of Flouret /1/ with some modification /2/. $(3,5\text{-Diiodo-Tyr})^2$-CCK-8 was synthesized on the solid phase by a standard procedure /3/. Tritiation of diiodo-vasopressins was performed in 0.1 M pH 6.0 phosphate buffer in the presence of Pd/Al_2O_3 catalyst /2/. I_2-CCK-8 was tritiated in dimethyl-formamide with PdO /4/.

After prepurification on a Sep-Pak C-18 cartridge, pure labeled peptides were isolated by RP-HPLC.

High-performance liquid chromatography

Purification of the tritiated vasopressins was carried out on an Altex HPLC apparatus, consisting of two pumps (Model 110 A), a System Controller (Model 420) and a Rheodyne injector (Model 7120), a μBondapack C_{18} column (3.9 mm x 30 cm) being applied. The purity of the separated peptides was checked on a Nucleosil 5 C_{18} column (4.6 mm x 25 cm). [^{3}H]-CCK-8 was purified with a Knauer HPLC system, on a Nucleosil 5 C_{18} column.

The column eluates were monitored for UV absorbance at 278 nm, and in each of the 0.5 ml fractions collected the ra-

dioactivity was measured with a Packard Model 3255 liquid-scintillation spectrometer.

All solutions were prepared using deionized and glass-distilled water, and passed through a 0.45 μm Millipore HA membrane filter. HPLC-grade methanol was used as supplied by E. Merck. The labeled vasopressin analogues were purified by gradient elution (components: 8% acetic acid solution and methanol), while isocratic chromatography (40:60 (v/v) 0.1 M pH 6.5 ammonium acetate buffer - methanol) was used for the isolation of [^{3}H]-cholecystokininoctapeptide and for analytical work. Just before chromatography, the mobile phases were degassed for 5 min in an ultrasonic bath.

The specific radioactivities of the tritiated peptides were determined via their UV spectra, amino acid analyses or radioimmunoassay.

The antidiuretic activities of [^{3}H]-AVP and [^{3}H]-dDAVP, the potency of [^{3}H]-dGAVP in memory tests, and receptor binding or [^{3}H]-CCK-8 on mouse brain homogenate were checked.

RESULTS AND DISCUSSION

Biological studies on peptide hormones demand high purity radiolabeled materials. Tritiated peptides can be purified by the same chromatographic methods as used for non-labeled substances. The long-established procedures such as paper, thin-layer, ion-exchange and gel chromatography have been applied for this purpose. However, their disadvantages (time-consuming, limited separating ability, loss of peptide, side-reactions during the process, etc.) make these methods inconvenient for the preparation of labeled compounds.

In contrast, high-performance liquid chromatography involves high resolving power, speed, simplicity and a good material recovery. Due to these features, HPLC has proved to be a useful tool for the purification of [^{3}H]-GnRH /5/, [^{3}H]-somatostatin /6/, [^{3}H]-substance-P /7/, [^{3}H]-TRF /8/, as well as of the [^{3}H]-$ACTH_{4-9}$ analogue, [^{3}H]-des-Tyr-γ-endorphin /9/ and [^{3}H]-gastrin analogues /10/.

The tritiation of 8-Lys-vasopressin and 8-Arg-vasopressin, and their purification by gel-permeation chromatography /11/ and affinity chromatography /12/ have been described.

We have isolated tritiated vasopressin analogues by reversed-phase HPLC. During the labeling of diiodo-vasopressin, [^{3}H]-(diAla^{1,6})-vasopressin with open ring, [^{3}H]-vasopressin and [^{3}H]-monoiodo-vasopressin were formed (Fig. 1.)

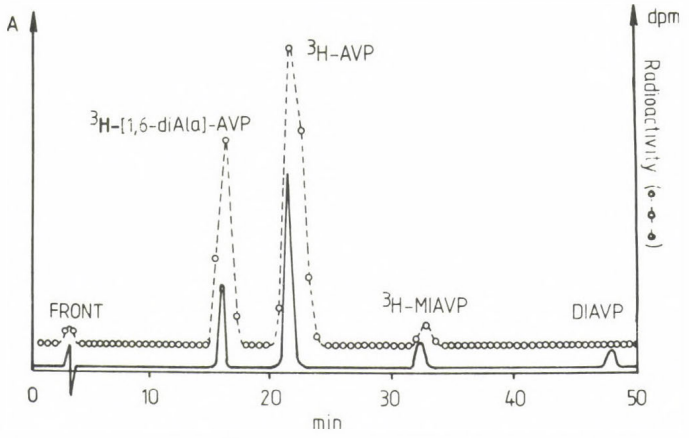

Fig. 1. Purification of [^{3}H]-AVP on a μBondapack C_{18} HPLC column. Elution was performed isocratically from 0 to 10 min (10% methanol in 8% acetic acid solution), following a linear gradient from 10 to 50% methanol (10-50 min), at a flow rate of 1 ml/min

The peptides were separated well and 80-90% of the injected radioactivity was recovered. The second chromatography of [^{3}H]-AVP, [^{3}H]-DDAVP and [^{3}H]-dGAVP on a Nucleosil 5 C_{18} column gave more purified peptides of high specific radioactivity. The biological activities of the tritiated hormone analogues are the same as those of corresponding non-labeled peptides (Table 1). The radioactive materials were stored in liquid nitrogen for two years and underwent negligible decomposition.

The synthesis and HPLC purification of [^{3}H]-labeled CCK-8 has so far been reported in a short communication /13/. We must emphasize the advantages of RP-HPLC in the preparation of this labeled peptide.

Table 1.

	[^{3}H]-AVP	[^{3}H]-DDAVP	[^{3}H]-DGAVP	[^{3}H]-CCK-8
SPECIFIC ACTIVITY	625 MBq/mmole 16.9 Ci/mmole	570 MBq/mmole 15.4 Ci/mmole	596 MBq/mmole 16.1 Ci/mmole	1554 MBq/mmole 42.0 Ci/mmole
PURITY ON HPLC	> 97%	> 97%	> 97%	> 97%
t_R VALUE	7.9 min	12.9 min	7.6 min	11.2 min
BIOLOGICAL ACTIVITY	397 IU/mg (96%)	928 IU/mg (97%)	93%	96%
STABILITY	After 2 years in storage in liquid nitrogen, decomposition is negligible			Degradable

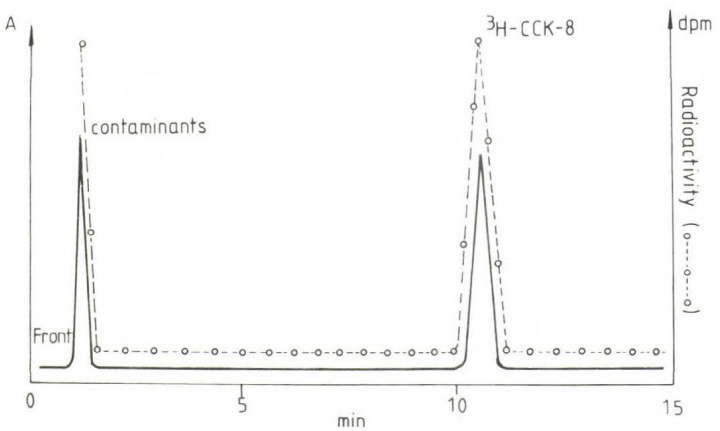

Fig. 2. Purification of [^{3}H]-CCK-8 on a Nucleosil 5 C_{18} HPLC column. Solvent system: 40:60 (v/v) ammonium acetate buffer (0.1 M, pH 6.5) - methanol. Flow rate: 1 ml/min

In spite of the presence of the sensitive amino acids Met and Trp, the rapidity of the process yields the pure peptide (Fig. 2) without any degradation which otherwise always occurs

with this tritiated hormone on TLC or by conventional column chromatography. The biological experiments must be carried out as soon as possible because [^{3}H]-CCK-8 is sensitive to oxidation and autoradiolysis, even when stored at -80 ^{o}C.

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SUBSEQUENT ANALYSIS TO PROTEOLYTIC DIGESTION OF SATIETIN-D

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SUMMARY

Satietin-D, an anorexigenic glycoprotein obtained from human plasma, was further purified by proteolytic digestion and the purification was followed by means of polyacrylamide gel electrophoresis (PAGE) in absence and in the presence of sodium dodecyl sulfate (SDS-PAGE). The amino acid composition of satietin-D, which proved to be homogeneous after tryptic-chymotryptic digestion and maintained its anorectic effect, was examined by amino acid analysis.

INTRODUCTION

Satietin and satietin-D are known as highly potent endogenous food-intake inhibitors which were isolated from human plasma (1, 2, 3, 4). Chemical analysis and preliminary characterization of these substances revealed that they are carbohydrate-rich glycoproteins (2, 3). Partial tryptic-chymotryptic digestion was the final step in the course of the isolation of satietin-D, which delivered a homogeneous product. The homogeneity of satietin-D was proved by polyacrylamide gel electrophoresis (PAGE), in the presence of sodium dodecyl sulfate (SDS) (SDS-PAGE) (4). The present paper demonstrates that satietin-D preserves its basic chemical and pharmacological properties after enzymatic treatment.

MATERIALS AND METHODS

Satietin-D was isolated from human plasma and subjected to tryptic-chymotryptic digestion as previously described (4).

SDS-PAGE SDS-polyacrylamide gel electrophoresis experiments were carried out on gel rods in a 12-tube gel electrophoresis cell (Reanal, Budapest) according to Laemmli's method (5). The total acrylamide concentration was 12.5%. 100µg loads of purified materials were used for electrophoresis and gels were stained with Coomassie brilliant blue R-250 (CBB R-250) as described by Nagy et al (4).

PAGE: The procedure of gel polymerization and the discontinuous buffer system used were as described by Davis and Ornstein (6,7). The experiments were carried out in the same cell as in the case of SDS-PAGE and the total acrylamide concentration varied over a wide range (T = 7.5-12.5%) at a constant cross-linking degree. Sample solutions containing 100 µg of satietin-D, together with 10% of glycerol and 0.001% of bromophenol blue, were layered on the top of the spacer gel before electrophoresis was started. Electrophoresis was performed at room temperature and a constant current of 2 mA/tube was maintained until the dye front approached the lower end of the gel tube. The gels were stained and destained as described previously (4).

Biuret method: Protein content of the samples was determined by microbiuret method as described by Honn and Chavin (8).

Amino acid analysis: Satietin-D samples subjecting for amino acid analysis were hydrolyzed at 110^{o}C in evacuated and sealed tubes with constant-boiling HCl for 24, 48 and 72 hours. Amino acid compositions were calculated by extrapolation to zero hour. For cystine analysis, the samples were oxidized with performic acid (9) and hydrolyzed. In addition to determine the tryptophane content, the samples were hydrolyzed in 4 M NaOH for 5, 10 and 20 hours at 105^{o}C. Amino acid analyses of the hydrolyzed samples were performed on a Bio-Cal, Model BC-200 amino acid analyzer using a single column (Aminex A-5) with a

two-buffer system: 0.2 M Na^+, pH 3.25, and 0.8 M Na^+, pH 4.25, sodium citrate (10).

Analysis of amino sugars were carried out under conditions mentioned above after 4 M HCl hydrolysis for 8, 13 and 20 hours at 100°C.

RESULTS

Fig. 1 shows the SDS-PAGE and PAGE of satietin-D before and after 24 h tryptic- chymotryptic digestion, which claimed to be the last step of isolation. It can be seen that the relative mobilities of major bands representing the active sub-

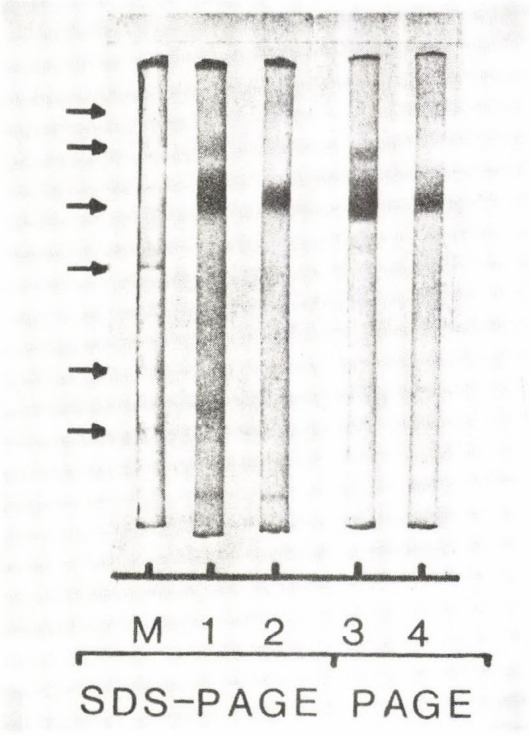

Fig. 1. 1 SDS-PAGE and PAGE of satietin-D before and after proteolytic digestion. M: Protein mixture from top to bottom: phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, lysozyme; 1,3: satietin-D before digestion; 2,4: satietin-D after digestion. The gels were stained with CBB R-250

Table 1. Protein content of satietin-D samples before and after digestion determined by biuret method and amino acid analysis

	Protein content of satietin-D	
	Before digestion % (w/w)	After digestion % (w/w)
biuret method	40.4	20.5
amino acid analysis	43.7	20.7

stances were not changed, but at the same time the impurities were removed by the digestion. The band of homgeneous satietin-D obtained by PAGE is wider and more diffuse than that of using the SDS-PAGE method, although this difference cannot be observed before the digestion step.

The Ferguson plots (11) of substances analyzed are shown in Fig. 2. The intercepts (Y_O) and slopes (K_R) of the curves of the molecules are similar.

The total protein content determined by the biuret method and amino acid analysis is listed on Table 1. The results obtained by these two different methods show similar values and the digestion process resulted in a 50% decrease in the total protein content.

Table 2 shows the amino acid composition of satietin-D before and after digestion. These values slightly differ from the results obtained previously (4) since then the calculations were carried out after 18 hours hydrolysis. Digestion resulted in an equal, about 50% decrease of the amino acid content, while the concentration of amino sugars showed a small, but significant increase, subsequently to amino acid analysis.

DISCUSSION

The appearence of a single band of satietin-D under the conditions of SDS-PAGE described above does not necessarily proves homogeneity. Therefore, it was advisable to make PAGE

Table 2. Amino acid composition of satietin-D samples before and after digestion

Amino acid	Before digestion	After digestion	
	(nmol/mg)	nmol/mg)	Nearest integer
Ala	216	104	4
Arg	115	39	2
Asp	302	161	6
$(Cys)_2$	53	29	1
Glu	322	218	8
Gly	192	114	4
His	52	26	1
Ile	139	65	3
Leu	280	98	4
Lys	692	280	11
Met	57	36	1
Phe	157	49	2
Pro	213	104	4
Ser	196	104	4
Thr	242	135	5
Trp	88	69	3
Tyr	154	75	3
Val	156	75	3
GalN	80	87	3
GlcN	349	369	14

measurements. However homogeneous satietin-D forms a single band in PAGE, but it shows up as a diffuse one. During digestion the relative molecular mass did not change since the K_R values calculated from Ferguson plots remained constant, while Y_o showed a minor decrease. According to these results a certain degradation may occur, but this change is presumably small.

The very large change (50%) in the protein content following enzymatic treatment can mainly be attributed to the removal of peptide contamination of serum origin. The total change concerning the whole amount of satietin-D is only 20%.

The digestion step removed 50% of the amino acid content of the substance, while left its biological activity uneffected and did not influence the molecular mass of the compounds as it is demonstrated by the measurements by SDS-PAGE (43,000) or amino acid analysis (39,500) (12). According to these findings satietin-D seems to be highly purified before the last isolation step, because it consists of at least 80% of the biologically active substance.

We can conclude from the analytical measurements described in this paper that the proteolytic digestion proved to be a useful method in producing homogeneous satietin-D, because the chemical characteristics of the biologically active substance have not changed definitely concerning electrophoretic behavior, relative molecular mass and biological activity.

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PURIFICATION OF IMMUNOLOGICALLY REACTIVE FRAGMENTS OF HUMAN CHORIONIC SOMATOMAMMOTROPIN BY PREPARATIVE REVERSED-PHASE HPLC

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INTRODUCTION

Human chorionic somatomammotropin (hCS) is a protein hormone of placental origin, composed of a single polypeptide chain of 191 amino acids with two disulfide bridges. The protein shows a remarkable homology with human growth hormone (hGH) (1).

We have recently undertaken a study of the antigenic structure of hCS, and have demonstrated that the synthetic peptide corresponding to the sequence 166-174 represents an antigenic determinant common to hCS and hGH (2). We have also begun to study the fragmentation of the hCS molecule, in order to obtain further information on the structural collocation of the different antigenic determinants. We have prepared hCS fragments derived from a nicked form of the hormone, after reduction and carboxymethylation (3), and furthermore, we have specifically cleaved the protein at the methionyl residues by treatment with cyanogen bromide.

In this paper we report the results regarding the preparation of highly purified hCS fragments using the HPLC technique. Preliminary results on the immunochemical characterization of these fragments are also reported.

EXPERIMENTAL

General methods

Electrophoresis on sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) was performed according to Swank and Munkres (4) using a gel with 12.5% acrylamide, and a bisacrylamide:acrylamide ratio of 1:15. The samples were dissolved in the sample buffer without β-mercaptoethanol. All SDS-PAGE chemicals were obtained from BioRad Labs. Molecular weight standards, i.e., myoglobin and the products of its cleavage by cyanogen bromide were obtained from Fluka.

For amino acid analysis, the samples were hydrolyzed at 110°C for 24 hr with twice glass-distilled 6N HCl containing 0.05% β-mercaptoethanol (Merck). The amino acid analyzer Chromakon 500 (Kontron) was employed.

Anti-hCS mouse immunoglobulins were obtained from hyperimmune sera produced in our laboratories and were purified on Protein A-Sepharose (Pharmacia)(5), eluting in a single step at pH 3.5. The immunoblotting experiments were performed by transferring the proteins from SDS-PAGE to nitrocellulose sheets (BA 85, from Schleicher and Schuell) as previously described by Towbin et al. (6). The nitrocellulose sheet was cut into 0.9 cm strips containing the transferred proteins and, after soaking in 3% bovine serum albumin in buffered saline (0.9% NaCl/10 mM TRIS.HCl, pH 7.4, with 0.05% Tween 20), the strips were exposed to the antibody solution overnight at 4°C. Finally, the strips were incubated for 2 h with peroxidase-conjugated rabbit anti-mouse IgG (Miles-Yeda) at 1/1000 dilution, and then treated with the enzyme substrate solution to visualize the antigen bands as described by Hawkes et al. (7).

Preparation of reduced and carboxymethylated hCS

The hormone was purified according to our published methods (8). Reduced and carboxymethylated hCS (RCOM-hCS) was prepared by reduction of purified hCS with dithiothreitol (Sigma) and subsequent alkylation with iodoacetic acid (Merck) (9).

Cleavage of hCS with cyanogen bromide

Cleavage at the methionine residues (10) was performed by treating hCS for 16 h with 70% (v/v) aqueous formic acid in the dark. A 50-fold molar excess of CNBr with respect to the methionine residues was added to the protein solution (about 10 mg/ml). At the end of the reaction the mixture was diluted with 10 volumes of water and freeze-dried.

Reversed-phase HPLC purification

A Perkin-Elmer HPLC system was used which consisted of a Series 3B, liquid chromatograph a model LC-75 variable wavelength spectrophotometric detector and a model 56 single-channel recorder. The detector operated at a wavelength of 210 nm. A μBondapack C_{18} (10 μm) column, 30 x 0.4 cm, was used (Waters). The purifications were achieved using gradients of acetonitrile or methanol (LiChrosolv, Merck) in 0.1% aqueous trifluoroacetic acid (Spectrosol, BDH).

Water used for the preparation of the solution of trifluoroacetic acid was purified on a Milli-Q system (Millipore) and subsequently passed through a Lobar Lichropep RP-8 column (24 x 1.1 cm, Merck). The mobile phase solutions were routinely filtered using 0.22 μm Millipore filters. Sample sizes varied between 300 and 1000 μg of peptide material injected in volumes of 100-150 μl.

RESULTS AND DISCUSSION

The primary structure of hCS is shown in Figure 1. The peptide bonds involving the carboxyl group of methionine residues, which are cleavable with CNBr, are indicated by arrows. An arrow also indicates the bond between residues 100 and 101, which proved to be cleaved in a nicked form of the hormone (3); another nicked form has also been identified in smaller amounts in the hCS preparations, but the point of cleavage has not yet been detected. The amounts of nicked forms vary con-

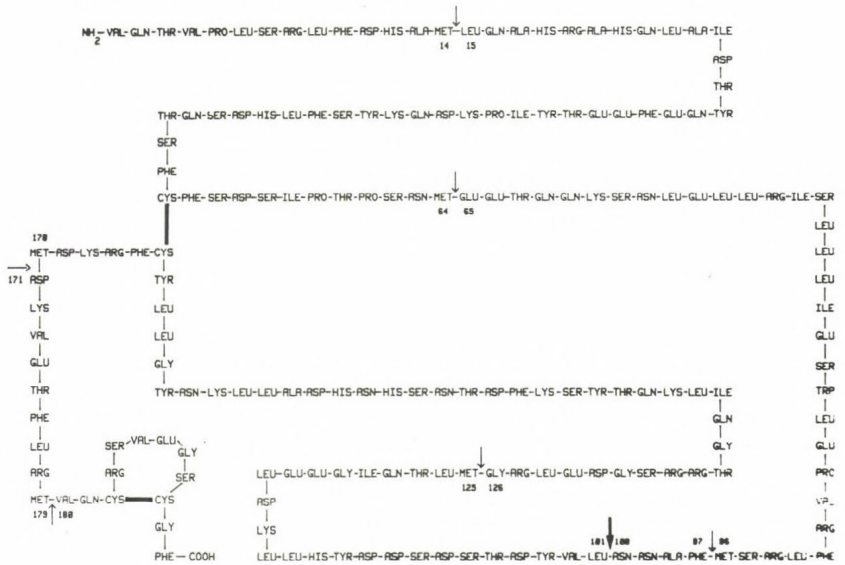

Fig. 1. Primary structure of hCS

siderably from one lot to another, but generally represent about 10-20%.

When the protein is reduced and carboxymethylated, the nicked forms give rise to the formation of four fragments. Figure 2 shows the electrophoretic profiles of hCS (A) and its carboxymethylated form (B), with the intact protein and the four fragments (α, β, γ, δ). As previously shown, fragments β and γ should correspond respectively to polypeptides 1-100 (M = 11800) and 101-191 (M = 10600) of hCS. α and δ should correspond to the secondary cleavage point of the molecule. The figure also shows that fragments α, β and γ, as well as the intact protein, reacted with anti-hCS antibodies.

Our experiments were aimed at the purification of the β and γ fragments which are present in larger amounts, in order to obtain a more detailed chemical and immunochemical characterization.

The first step in the separation of these fragments was gel filtration on Sepharose CL 6B in 6M guanidine hydrochloride. The sample is not completely soluble in non-denaturing solvents

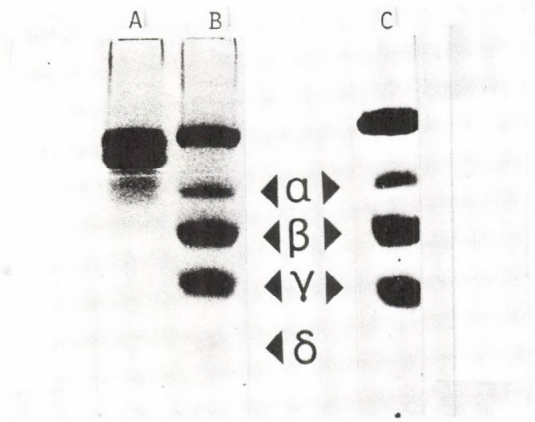

Fig. 2. Electrophoretic and immunochemical analysis of the fragments from RCOM-hCS
Lane A: SDS-PAGE of hCS (10 µg) showing the intact form (fast band) and the nicked form (slow band) of the protein
Lane B: SDS-PAGE of RCOM-hCS (30 µg).
The apparent molecular weights of fragments α, β, γ and δ, estimated from the electrophoretic mobilities, were respectively 16,000, 12,200, 8,800 and 5,900 as estimated from their electrophoretic mobilities (3)
Lane C: immunoblotting of RCOM-hCS (4 µg) performed with mouse anti-HCS antibodies

and usually the denatured molecules are excluded from gel media that normally admit the native form (11).

The chromatographic profile obtained by loading RCOM-hCS on the column shows two main asymmetrical peaks (Figure 3). Six pools were formed from the fractions and were analyzed electrophoretically. Fraction "E" is of particular interest because it contains only fragments β and γ. As this fraction represents about 20% in weight of the RCOM-hCS sample, fractions "E" derived from four separate chromatographic separations performed under the same conditions were pooled in order to work with a more manageable amount.

The two components of fraction "E" were separated on the basis of their different solubility in 0.01N NH_4HCO_3. When fraction "E" was suspended in 0.01N NH_4HCO_3, a soluble fraction containing the γ fragment with a small amount of β fragment

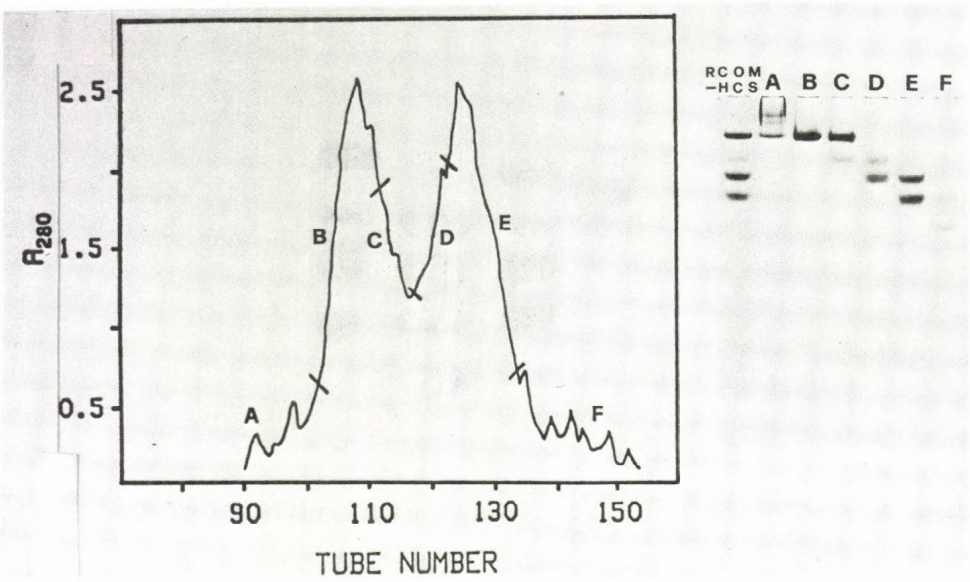

Fig. 3. Gel chromatography of RCOM-hCS on Sepharose CL-6B in 6M guanidine hydrochloride. Column: 2.5 x 87 cm. Flow rate: 6.7 ml/h. Detector: U.V. 280 nm. Sample loaded: 20 mg of lyophylized RCOM-hCS
Six pools (A, B, C, D, E, F) were collected by combining fractions as indicated. The figure also shows the SDS-PAGE patterns of the pools collected, compared to the original sample

Fig. 4. Electrophoretic analysis of samples obtained by fractional solubilization of pool "E" from gel filtration of RCOM-hCS
Lane 1: Pool "E" (10 µg)
Lane 2: Insoluble fraction (10 µg)
Lane 3: Soluble fraction (10 µg)

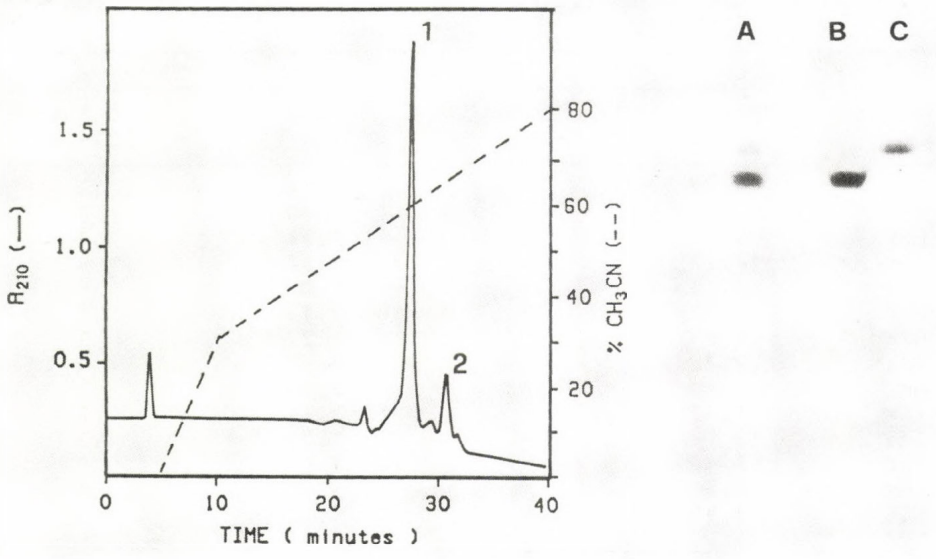

Fig. 5. Reversed-phase HPLC purification of the fragment from RCOM-hCS. The starting solvent was 0.1% trifluoroacetic acid in water, and a gradient of acetonitrile was employed as indicated by the dotted line. The chromatogram refers to a semipreparative separation, where 340 µg of the sample were loaded onto the column. The figure also shows the SDS-PAGE patterns of:
Lane A: Soluble fraction from fractional solubilization (8 µg).
Lane B: Peak 1 (5 µg)
Lane C: Peak 2 (5 µg)

was obtained, while the precipitate was composed of highly purified fragment β (Figure 4).

We obtained a complete purification of γ fragment with the HPLC technique using a C_{18} column and a gradient of acetonitrile in 0.1% trifluoroacetic acid (Figure 5). In our chromatographic conditions we obtained two main peaks by loading onto the column the soluble fraction obtained from the previous experiment. The electrophoretic analysis shows that peaks 1 and 2 were composed of fragments γ and β, which proved to be well separated.

The homogeneous fractions β and γ were analyzed for their amino acid content (Table 1). This analysis shows that the two fragments in fact correspond to sequences 1-100 and 101-191 of

Table 1. Amino acid composition of β and γ fragments purified from RCOM-hCS

Amino acid	β		γ	
	Found	Calculated 1-100	Found	Calculated 101-191
Asx	9.70	9	16.04	13
Thr	5.82	6	3.40	6
Ser	9.74	11	6.44	7
Glx	16.02	16	9.22	9
Pro	4.25	5	N.D.	0
Gly	1.39	0	7.40	7
Ala	4.65	5	1.27	1
Val	2.75	3	3.57	4
Met	1.20	3	2.79	3
Ile	4.27	5	2.11	2
Leu	13.20	13	11.70	12
Tyr	3.46	3	4.61	5
Phe	6.56	7	3.91	4
His	3.97	4	3.07	3
Lys	3.74	3	6.15	6
Arg	5.16	5	6.14	6
Trp	N.D.	1	N.D.	0
CM-Cys	N.D.	1	3.36	3

Table 2. Amino acid composition of peak No. 6 from HPLC of CNBr-cleaved hCS

Amino acid	Found	Calculated 15-64-SS-126-170
Asx	12.00	12
Thr	6.70	7
Ser	9.15	9
Glx	11.27	11
Pro	N.D.	3
Gly	3.82	4
Ala	3.86	4
Half-Cys	1.02	2
Val	1.65	0
Ile	3.51	4
Leu	9.26	9
Tyr	5.65	6
Phe	6.37	6
His	5.23	5
Lys	6.18	6
Arg	5.46	5

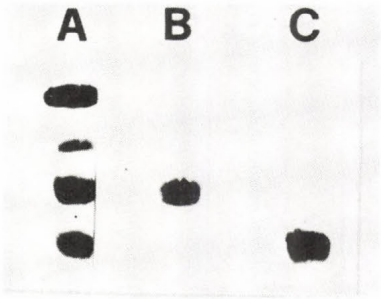

Fig. 6. Immunoblotting of the and fragments purified from RCOM-hCS, performed with mouse anti-hCS antibodies
Lane A: RCOM-hCS (4 µg).
Lane B: fragment (2 µg)
Lane C: fragment (2 µg)

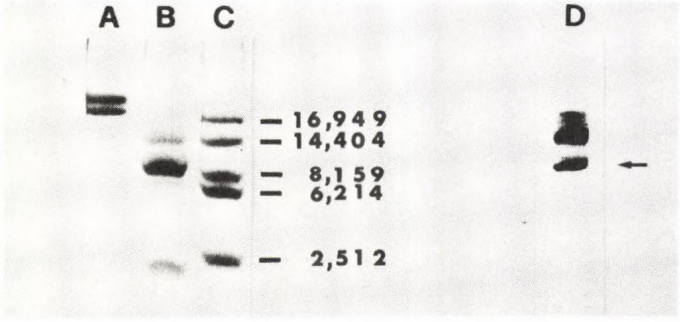

Fig. 7. Electrophoretic and immunochemical analysis of the fragments from CNBr-cleaved hCS
Lane A: SDS-PAGE of hCS (8 µg)
Lane B: SDS-PAGE of the reaction products of CNBr-cleaved hCS (30 µg).
Lane C: SDS-PAGE of the molecular weight standards
Lane D: immunoblotting of CNBr-cleaved hCS (20 µg) with mouse anti-hCS antibodies

hCS, thus confirming a main cleavage of the molecule at the level of the Asn-Leu bond, 100-101.

The immunoreactivity of the purified fragments was tested by immunoblotting. Figure 6 shows that, even after the purification procedure, the β and γ bands react with the specific antibodies.

As far as the results of hCS fragmentation with CNBr are concerned, it can be observed that there are six methionines in

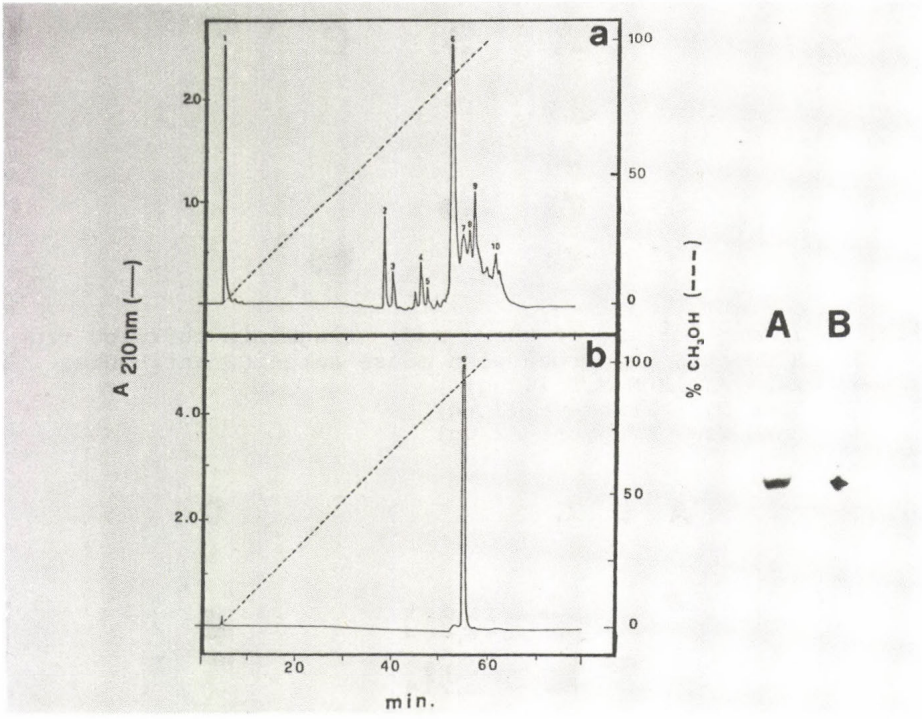

Fig. 8. Reversed phase HPLC of CNBr-cleaved hCS. Linear gradient in 60 min obtained with initial eluent 0.1% TFA and final eluent methanol
a) Chromatographic profile obtained by loading onto the column 1 mg of the reaction product of hCS with CNBr
c) Chromatographic profile of peak 6 from pooled fractions (800 μg). The figure also shows the electrophoretic analysis (lane A) and the immunoblotting performed with mouse anti-hCS antibodies (lane B) of the purified chromatographic fragment

the hCS sequence, so that seven fragments are theoretically predictable. However, the S-S bond between cysteins 53 and 165 held together two polypeptides in a single fragment, 15-64-SS-126-170, with molecular weight 11,000.

The reaction product of hCS with CNBr was analyzed by SDS-polyacrylamide gel electrophoresis and by immunoblotting with anti-hCS antibodies in order to test the immunoreactivity of the fragments (Figure 7). We observed a marked positive reaction corresponding to the electrophoretic band of molecular

weight about 11,000 (indicated with an arrow), which should correspond to the peptide fragment 15-64-SS-126-170. Reactions can also be seen with bands of molecular weight over 11,000; these can be attributed to the presence of fragments which are only partially attacked by cyanogen bromide, or to polymerization phenomena.

The hCS fragments derived from cleavage with CNBr were separated by a single step of HPLC (Figure 8a). We used a reversed phase system with a linear gradient of methanol in 0.1% trifluoroacetic acid. The peaks were collected, dried under nitrogen and analyzed by electrophoresis to identify the peak corresponding to the fragment of molecular weight 11,000. It proved to be peak no. 6. A pool of the material corresponding to peak no. 6, collected from several chromatographic separations, was rechromatographed under the same conditions (Figure 8b). The electrophoretic analysis of this material demonstrated its high degree of purity. Moreover, the fragment obtained again showed a positive reaction with anti-hCS antibodies when tested by immunoblotting.

The amino acid analysis (Table 2) showed that peak no. 6 did in fact correspond to the fragment composed of peptides 15-64 and 126-170, linked by the disulfide bridge between cysteines 53 and 165.

Our experiments have demonstrated that the HPLC technique can be used successfully for the purification of immunologically reactive peptide fragments. In the case of CNBr fragments, the peptide of interest presented good solubility in aqueous trifluoroacetic acid, which allowed its purification with a single HPLC step. In the case of peptides derived from nicked hCS, however, fragment β was not sufficiently soluble in the solvents commonly adopted for HPLC of peptides. A preliminary step of gel chromatography in 6M guanidine hydrochloride, followed by a fractional solubilization in 0.01N NH_4HCO_3 led to the purification of fragment β and yielded a mixture enriched in fragment γ, which could thus be efficiently purified by HPLC.

The purified peptide fragments all proved reactive with specific hCS antibodies, when analyzed by the immunoblotting technique. A quantitative analysis, for example by radioimmuno-

assay, will, however, be necessary for a more complete evaluation of the immunochemical reactivity of the isolated peptide fragments, in comparison with that of the intact protein. A more complete characterization of the antigenic determinants of the peptide fragments is in progress, using some available hCS monoclonal antibodies (12).

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H. Kalász and L.S. Ettre (Eds)

HPLC OF NUCLEOTIDES. III. PURPOSEFUL CONTROL OF SELECTIVITY IS THE APPROACH TO PERFECT AND INFORMATIVE CHROMATOGRAPHY

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Our earlier investigations /1, 2/ in HPLC of nucleotides including normal and reversed-phase, ion-exchange and ion-pair chromatography were continued. They were mainly focused on the determination of selectivity, on the factors influencing it and on the methods of selectivity control for the further development of these chromatographic methods. The proper determination of selectivity is required for the objective evaluation of adsorbents and chromatographic procedures. The knowledge of factors effecting selectivity allows to optimize separation, while the purposeful control of selectivity allows to make chromatography as much informative as being able to identify the component of interest in the course of separation. We are investigating the above mentioned problems for the development of HPLC of nucleotides, but they are also actual for any other types of polyfunctional compounds.

EXPERIMENTAL

The procedures for column packing, treatment before use, testing as well as for separations of nucleotide mixtures were described previously /1, 2/. The following columns were used: Ultrasphere (Du Pont) and Ultrasil (Altex) Beckman), Nucleosil (Macherey-Nagel).

THE MAIN PARAMETER OF THE SEPARATION SYSTEM.
THE CONDITIONS FOR PROPER DETERMINATION

The resolving power (R_s) is governed by three major parameters that is efficiency (N = number of theoretical plates), retention (k' = capacity factor = $(t_R - t_o)/to$) and selectivity (α = coefficient of selectivity = k'_2/k'_1) which are involved in one equation (1):

$$R_s = \frac{\sqrt{N}}{4} \left(\frac{k'}{k'+1}\right) \frac{(\alpha-1)}{\alpha} \qquad (1)$$

The priority of α can be easily demonstrated by a simple arithmetic example. A column with N = 10000, at k' = 1 and α = 1.06 has R_s = 0.75, which is sufficient for quantitation of the chromatogram. For the preparative separation an R_s value of 1.5 is necessary. This may be achieved by the increase of N up to 40,000 (that leads to an increase in both the analysis time and back-pressure) or by huge increase in retention ($\infty/(\infty+1)=1$), or, finally, by the enhancement of the selectivity coefficient up to 1.12 that is by only 5.5%.

The selectivity term is quite evident and unambiguous but its expression as the ratio of the component retentions does not represent unequivocally the selective properties of the system: it fails to possess the independence on the analytical conditions. We found that the capacity factor of nucleotides on a RP-column is linearly related to the eluent concentration in a log-log plot:

$$\log k' = a - b \log[MeOH]$$

and

$$\log k' = c + d \log[NH_4OAc]$$

(in the concentration range of 0.02-0.2 M), but as it was found earlier (see figure 16, page 451 of /2/) these lines are not parallel for various nucleotides. Therefore the ratio k'_2/k_1' is not constant at various eluent concentrations and the α-value is not constant either.

Taking into account the multiplicity of the chemical and physico-chemical factors effecting homo- and heterogeneous interactions in the eluent/adsorbate/adsorbent system, the wide use of a few adsorbents in modern HPLC might be the most surprising. But, at the same time, great variations in the selectivity of similar adsorbents from different manufactures to the same type of compounds were found.

How can different adsorbents be compared by selectivity to certain substances? We try to make this comparison under certain conditions (Fig. 1).

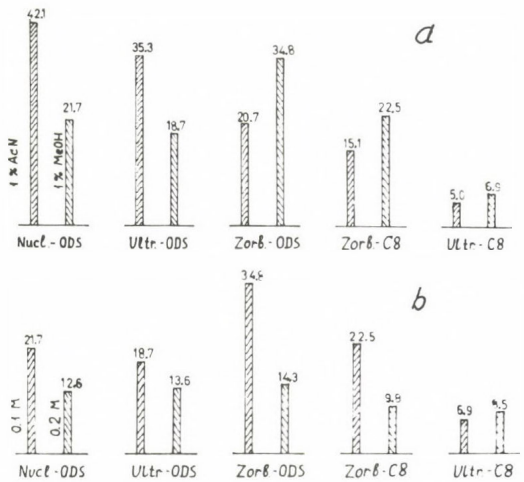

Fig. 1. Selectivity of RP-columns to mononucleotide separation ($\alpha_{dpA/dpC}$) in
a) 1% AcN or 1% MeOH buffered with 0.1 M NH_4OAc, pH 7.2;
b) in 1% MeOH buffered with 0.1 M or 0.2 M NH_4OAc

However, the study of selectivity at variable eluent concentrations demonstrates the functional dependence between retention and selectivity (Fig. 2).

Therefore we concluded that the tests of the adsorbents at a certain eluent concentration may properly demonstrate only their retention power. However, in order to compare adsorbents

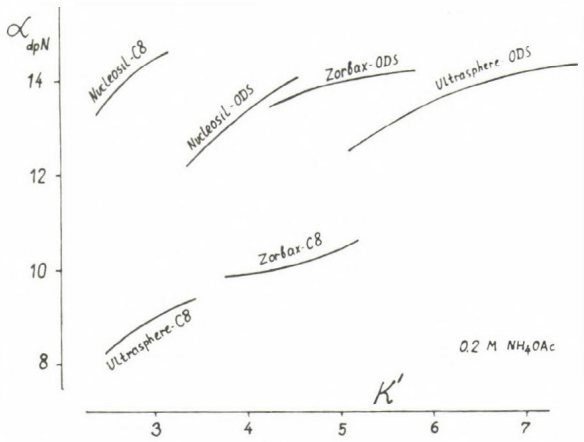

Fig. 2. The dependence of selectivity on changes in retention. Mobile phase: different concentrations of H_2O-MeOH buffered with NH_4OAc

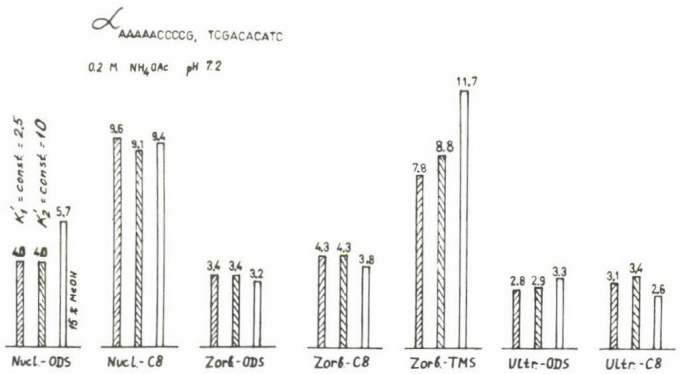

Fig. 3. Selectivity of some RP-columns to deoxyoligonucleotide separation, for a certain k' value (k' is constant for the first or second component) (left and center plots), and for a certain eluent concentration, (right plots)

in their selectivity it is necessary to determine it at equal retention for all adsorbents (Fig. 3).

These determinations of α under constant k'_1 or k'_2 almost always conincided and the right comparison appears to be per-

formed at a certain k'_2 comparing packings at equal productivity in respect to time.

The comparison at constant k' allows also to find out the sensitivity of the adsorbents, that is of the various stationary phases (C_1 - C_{18}), to the changes in the mobile phase concentration. The found dependence of the eluent concentration on the size of the radical of the stationary phase (/2/ page 449), allows to choose the preferable set of adsorbents. This is especially true for the substances differing in polarity and hydrophobicity, since they allow to move from one phase to another independently on the properties of the substances under separation and to perform separation in equally optimal conditions. For data about the optimization of RP-HPLC selectivity on pH, mobile phase composition and two components gradient program see /2/.

CONTROL OF SELECTIVITY. THE DEFINITION AND THE REASONS FOR APPLICATION

Up to now we did not define what kind of selectivity we keep in mind. As for nucleotides they differ in both sizes of the chains (the number of monomer units) and in the heterocyclic composition and sequence. The analysis of the selectivity of the adsorbents to oligonucleotides at a given k'-value clearly manifests the difference in the nature of nucleotide interaction with alkyl and aminoalkyl bonded phases. It had been found that for aminoalkyl adsorbents a much greater selectivity is observed to the oligonucleotide size (α = 1.8-2.3) according to ion-exchange mechanism than for alkyl adsorbents (α = 1.1-1.3) where it is due to molecular dispersive interactions.

The main difference of these groups exceeds 80% (for details see page 445, /2/). High selectivity of ion-exchangers to nucleotide charged groups may be realized when the molecular interaction of compositionally different isoplytes will not mask the ion-exchange picture. As we have demonstrated earlier, the selectivity of amino-phase adsorbents to nucleotide heterocycles is about 10 times lower than in the case of RP-

sorbents; however, for rather long oligomers their difference in the composition is manifested in marked difference in retention. The isoplytes are separated and the wider the time windows for each isoplyte type, the less clear becomes the dependence of retention on its length. Taking into account that the resolving power of commercial anionites to the separation of homooligomers allows to separate up to 20-22 homologues, it is clear that time windows for already 10-12 membered oligonucleotides with hetero-composition are almost completely overlapped.

The selectivity of aminoalkyl-silicas to heterocycles is managed to be greatly decreased, since the retention of nucleotides is strongly dependent on the pH of the mobile phase and for any anionite, one can found a pH-value which provides the lowest selectivity to the nucleotide composition /2/. We also hoped to suppress the molecular interactions by means of increasing the concentration of the organic solvent, but the opposite effect was found. It can be explained either by incomplete modification or shielding of the silanol surface of the silica or by changing the spatial structure of the stationary phase aminoalkyl radicals in aqueous-organic medium. We have already reported about the production of a new silica-based anionite, different from the commercial stationary bonded phases. Due to its high selectivity to the oligomer size and fast dynamics of mass-transfer our adsorbent allows to separate up to 25-30 oligomers in 1 hour and is capable to resolve up to 50-membered oligonucleotides. For providing purely ion-exchange HPLC it is also important that its selectivity to molecular interactions can be easily controlled: the addition of 50% methanol to the eluent (salt gradient) almost completely excludes the separation of isoplytes with various heterocyclic composition. The possibility to control selectivity leads to markedly better selectivity to the oligomer, allowing to obtain calibration "size-retention" for rather large oligonucleotides (Fig. 4). These dependences were obtained in the real conditions of the long-term use during isolation of the large number of oligonucleotides of interest and they allow to identify them rather reliably.

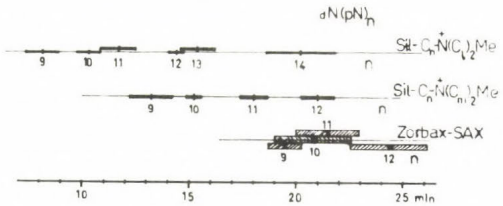

Fig. 4. "Time-length" correlation of oligonucleotides on Zorbax SAX and domestic anionite columns. Gradient elution: 0.4-2 M NH_4OAc/100 min (Zorbax SAX) and 0.15-1 M NH_4OAc/100 min (new anionites); 1 ml/min; 25°C

For clear distinguishing of the mass-transfer mechanisms of molecular and ion-exchange one can realize the earlier concept proposed by us about the need to separate the complex polyfunctional compounds by a set of highly specific methods, i.e., to use only one mechanism in each dimension.

For nucleotides as for many other compounds the two-dimensional separation is sufficient for complete and guaranteed homogeneity of the products and for their identification. In the first dimension (Fig. 5) by ion exchange-HPLC we achieve incomplete separation of the mixture but distinct separation according to the size and the isoplytes isolated at the expected retention are separated according to the composition in the second dimension by RP-HPLC.

For two-dimensional HPLC it is convenient to use a 3- or 4- component chromatograph expanding it with some components (Fig. 6). The gradients of the salt, the solvent and other modifiers are created separately by pump and valve systems. Instead of a dynamic mixer we include a precolumn (dead volume less 0.3 ml) which affords efficient mixing, additionally filters the eluent, and adsorbs the impurities present. The guard-column (after injector) has the usual function and a three-port valve allows to often wash both precolumns and regenerate them. We demonstrated earlier /2/ the use of two precolumns and that the use of a 3-way valve between the guard-column and the analytical column leads to a band broadening of not more than 3% upon gradient elution if the adsorbent used in

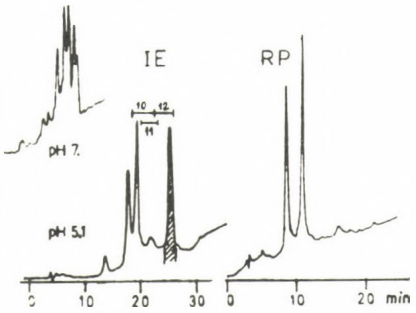

Fig. 5. Two-dimensional HPLC of a mixture of deoxynucleotides for the isolation of dodecanucleotides of interest by ion exchange chromatography on Zorbax NH_2 (compare at pH 7 and pH 5.1) and by reversed-phase chromatography of the isolated isoplytes on Zorbax C8

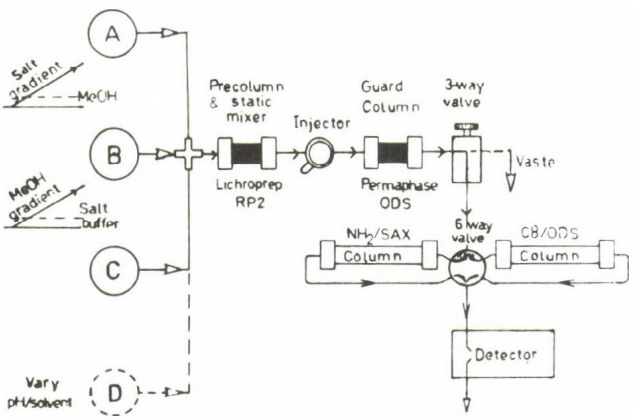

Fig. 6. The schematic of 3-or 4-component gradient chromatograph for two-dimensional HPLC. A, B, C(D) - pumps or time-proportional valves. The precolumn is introduced before injector instead of a dynamic mixer and filter; the 3-way valve is installed after the guard-column for the regeneration of the precolumns; the 6-port valve is used for attachment of two columns and the detector

the guard- and precolumn is selected properly. Finally, two analytical or preparative columns connected to the detector are attached by means of a 6-port Rheodyne-7000 valve.

The last example of selectivity control is in the field of normal-phase (NP) adsorption HPLC. It is widely used in the chemical synthesis of oligonucleotides (the so-called phosphotriester method) when all polar groups (including phosphate) of the nucleotides are protected in the intermediate stages. NP-chromatography is rather selective to nucleotide composition, to the presence or absence of all protecting groups, and also to the difference in spatial structure of the molecules since these derivatives - phosphotriesters - are diastereomeric relatively to each protected phosphate group. The separation of diastereomers leads to serious difficulties in the course of product purity analysis while their separation is not necessary since after removal of the protecting groups the isomery is vanished. In order to analyze the homogeneity and for preparative isolation of the protected blocks it is necessary to overcome this selectivity, retaining the selectivity to protective groups and to the heterocyclic composition.

The left side of figure 7 shows the superimposed chromatograms of the diastereomeric dimer with various combinations of the protecting groups. We propose that in this case one should use simultaneously the decrease in the retention power due to a decrease of the specific surface area of the silica adsorbent with larger pores and their better accessibility. We applied for this work a set of ArmSorb-Si columns (25 cm x 4 mm), efficiency 17,000-20,000 theoretical plates, packed with 5 μm silica with pore size of 60, 100 and 300 Å. The silica particles were irregular but were rounded by a special technology and thus had a spheroid shape. Their pore structure was formed by regular hydrogel microparticles. (These adsorbents and columns were developed with our participation in the Yerevan Department of Inorganic Materials of the All-Union Research Institute of Specially Pure Chemical Reagents. The method of the column packing is described in /2/).

As expected at equal elution conditions, the larger the pores and the lower the specific surface the less the retention (Figures 7 and 8). However in the case of elution with equal retention of the first isomer we obtained the expected decrease in the selectivity to isomery for an adsorbent with large pores:

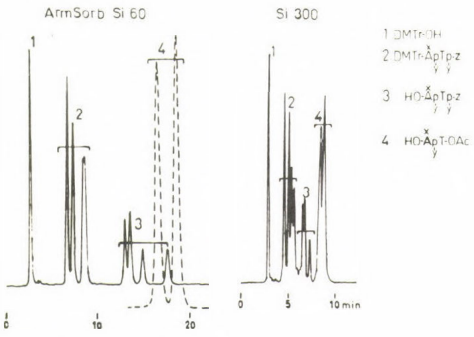

Fig. 7. The chromatograms of protected dinucleotide in 97:3 chloroform-methanol (methanol contained 7.5% water for isohydric conditions). DMTr-dimethoxytrityl, x-benzoyl, y-pCl-phenyl, z- CNEt

but, at the same time the group selectivity, i.e., selectivity to the type of functional group, increased.

As illustrated in Fig. 8, an increase in the selectivity of separation can be seen according to the type of functional group, i.e., between the last isomer of the first group and the first isomer of the second group.

	ArmSorb Si 60 Å		Si100 Å		Si 300 Å	
	K'	α	K'	α	K'	α
DMTr-ÀpTp-z	2.56	>1.46	2.27	>1.47	1.27	>1.59
	3.74		3.34		2.02	
HO-ÀpT-OAc	6.8	>1.15	4.65	>1.12	3.73	>1.12
	7.8		5.2		4.2	
	0.69	>3.18	0.58	>2.78	0.65	>1.45
	2.19		1.61		0.94	
				>1.98		>2.2
	2.12	>1.16	3.19	>1.13	2.07	>1.11
	2.46		3.61		2.3	

Fig. 8. Retention and selectivity data in NP-HPLC of diastereomeric derivatives of deoxydinucleotide AT, on silicas with different pore sizes. Upper data: elution at fixed eluent concentration, 97.5:2.5 chloroform-methanol.
Lower data: chloroform-methanol ratio of 97:3 (Si60), 98.2:1.8 (Si100) and 99:1 (Si300). Symbols of protective groups as in Figure 7

CONCLUSION

We have defined a method for selectivity estimation and demonstrated using certain examples how it works. This involves the suppression of selectivity to molecular composition for pure ion-exchange HPLC, the determination of the highest selectivity to oligomer composition in the course of reversed-phase HPLC and the suppression of selectivity to spatial structure in preference to increase in group selectivity to functional groups in normal-phase HPLC. These approaches may also be applied to other types of polyfunctional compounds. We hope that this approach leads to a more perfect and informative HPLC.

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SEPARATION OF SUBSTANCES OF VARIOUS CLASSES

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H. Kalász and L.S. Ettre (Eds)

LIQUID CHROMATOGRAPHIC DETERMINATION OF PLASTICIZERS USED IN PLASTIC INDUSTRY

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**Graboplast Cotton Weaving and Artificial Leather Producing Factory, Győr, Hungary

Determination of various phthalate ester plasticizers with reversed-phase high-pressure liquid chromatography (HPLC) is discussed for the purpose of the quality control of commercial products and identification as additives in plastics. The retention dependance of the phthalates is investigated on three different alkyl stationary phases as a function of the carbon number of their alkyl groups. It is shown that the log k' value of the individual components is monotonously increasing with the increase of the carbon number but it is not linear on C_8 and C_{18} stationary phases while being approximately linear on a C_2 phase.

The applicability of HPLC and gas chromatography (GC) for the determination is studied. Both methods are suitable for the investigation of phthalate plasticizers. Di(ethyl-hexyl) and octyl phthalates cannot be separated by GC on a packed column (SE-30) while they can be well separated with HPLC.

An additional advantage of HPLC is that it gives a good separation, even in a wide concentration range of the phthalates. At the same time the analysis can be accomplished in a shorter time by GC.

INTRODUCTION

The wide application of plastics involves is accompanied by the rise of pollution problems caused by them. These problems are very far-reaching because of the great variety of the

plastics, and because of their wide application fields. In the mid-seventies pollution caused by the phthalates became the subject of detailed investigations. The toxic effects of these polluting materials, mainly of their metabolites, on living organism have been established.

In the literature we found primarily reports on the HPLC determination of phthalates. Japanese authors /1, 2/ elaborated the identification of dibutyl phthalate and di(ethylhexyl phthalate in various waters. American authors /3/ demonstrated the determination of the metabolites of phthalates with HPLC and GC.

Takeuchi et al. /4/ reported on the determination of phthalates by supercritical fluid chromatography, using hydrocarbons as the whole phase.

No reference was found on the use of HPLC for the investigation of commercially produced phthalates applied as plasticizers. It seems to be obvious from the literature that HPLC can be used for the quality control of the phthalate plasticizers and for their detection in polyvinyl chloride (PVC) polymers. In such an application one must take into account that a wider range of phthalate homologs and isomers may be present and also that substances other than phthalates may be encountered during production.

Such investigations can be carried out by gas chromatography but high (around 300^{o}C) temperatures must be used and the higher dialkyl phthalates (C_{10}, C_{11} etc.) will elute only with difficulty.

EXPERIMENTAL

The following liquid chromatographic system has been used in our work:

An Orlita membrane pump complete with a pulsation damping unit, a Rheodyne 7105 injector, a variable wavelength UV spectrophotometric detector, a Spectra Physics Minigrator for data evaluation and a Speedomax recorder to register the chromatograms.

The experiments have been carried out using the following LC columns:

LiChrosorb RP-18 (Merck), d_p = 10 µm;
Column dimensions: 30 cm x 4 mm.

Nucleosil-10 C_{18} and C_8 (Macherey-Nagel),
d_p = 10 µm; column dimensions: 30 cm x 4 mm.

LiChrosorb RP-2 (Merck), d_p = 5 µm; column,
dimensions: 15 cm x 4 mm.

Various mixtures of water with methanol were used as the mobile phase. Their composition is given in the figures. The methanol was of analytical grade while the water was ion exchanged and distilled. Every solvent was filtered on a 5 µm sinter filter.

The samples were dissolved in methanol, in the concentration of 0.05 mg/ml.

RESULTS AND DISCUSSION

The separation of phthalates has been investigated on three different stationary phases. The individual stationary phases differed in the length of the alkyl chain: silica gel with bonded octadecyl, octyl and ethyl groups has been used.

Figure 1 presents the separation of dialkyl phthalate standards on octadecyl (RP-18) phase. The C_9 peak is the mixture of several dinonyl isomers which could not be separated into the individual components.

Figure 2 illustrates the investigation of a commercial plasticizer made in Hungary under the above-mentioned conditions. In the figure, the unmarked peaks correspond to the branched-chain or non-symmetrical alkyl esters of the neighbouring dialkyl phtalates. The unesterified phthalic acid residue is separately marked, identified as phthalic anhydride.

Figure 3 shows the separation of dialkyl phthalates on an octyl phase (C_8) while figure 4 demonstrates the investigation of three Hungarian-made plasticizers. The individual components are identified by the carbon number of the alkyl chains.

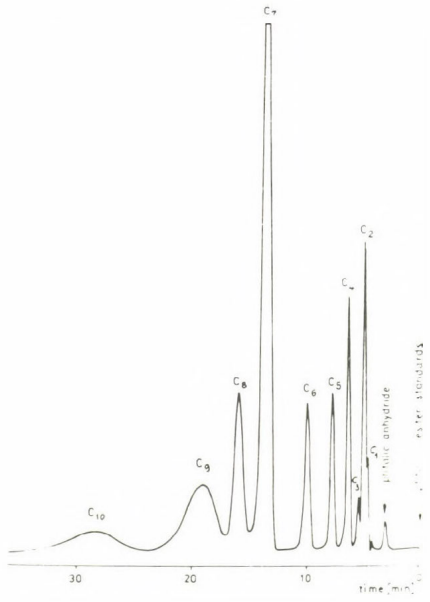

Fig. 1. Separation of phthalate standards by reversed-phase HPLC.
Column: 0.3 m x 4 mm. LiChrosorb RP-18, d_p = 10 µm
Eluent: 93:7 methanol/water. Detector: UV at 254 nm

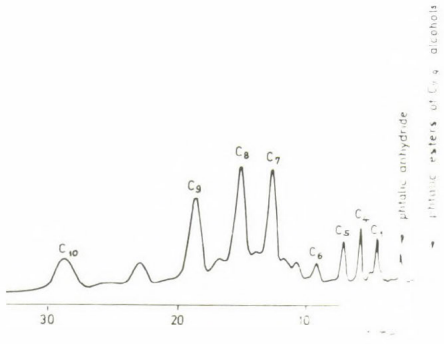

Fig. 2. Separation of a commercial plasticizer.
Conditions: as in Fig. 1

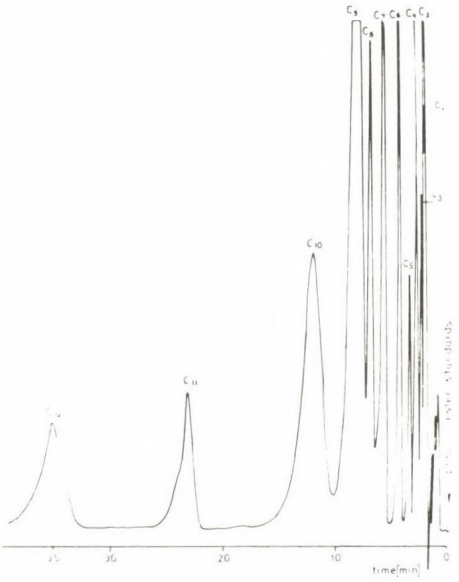

Fig. 3. Separation of phthalate standards.
Column: 0.3 m x 4 mm
Nucleosil-10-C_8, d_p = 10 µm
Eluent: 85:15 methanol/water, Detector: UV at 254 nm

The phthalate standards and the plasticizers have also been investigated on a bonded ethyl phase (Fig. 5).

The retention behaviour was investigated by the relationship of the logarithm of the capacity factor (k') on the carbon number of the alkyl chain in the phthalates (Figs. 6, 7, 8).

Increasing the length of the alkyl chain in the phthalates, the retention of the individual components is monotonously increasing; however, an approximately linear relationship has only been found on the RP-2 phase. The resolution value of the individual components depends primarily on the composition of the eluent, the influence of the composition of the stationary phase being less characteristic.

We have studied this relationship in order to find out whether the unknown components of the phthalate plasticizers can be identified having only a limited number of standards. Since the log k' vs. carbon number relationship is not comple-

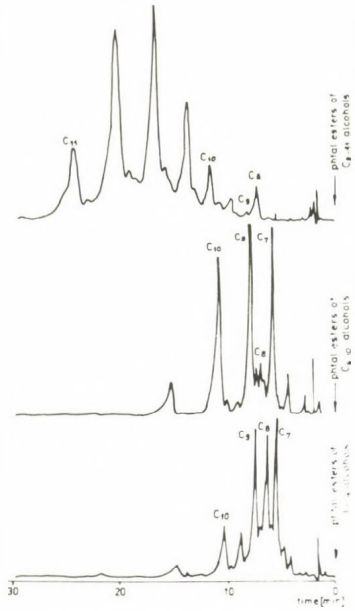

Fig. 4. Separation of some commercial plasticizers. Conditions: as in Fig. 3

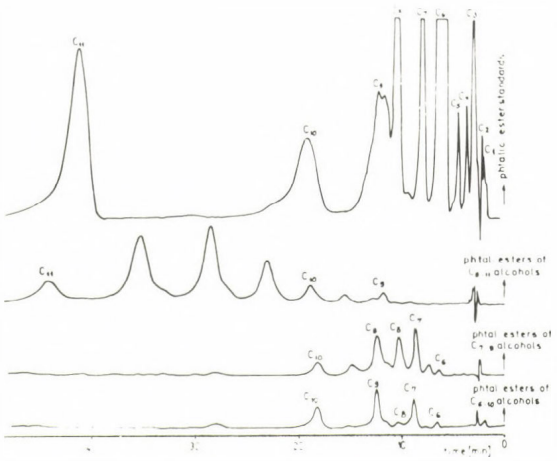

Fig. 5. Separation of phthalate standards and some commercial plasticizers.
Column: 0.15 m x 4 mm, LiChrosorb RP-2, d_p = 5 µm
Eluent: 85:5 methanol/water. Detector: UV at 254 nm

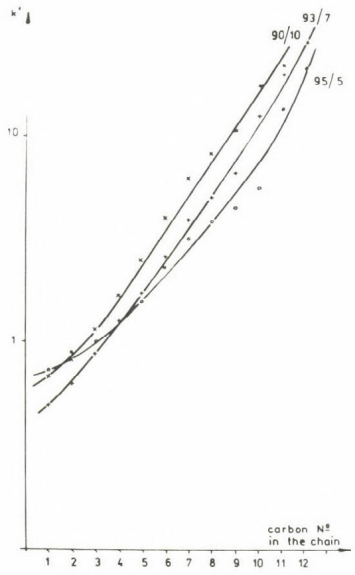

Fig. 6.
Relationship between log k' and the carbon number of the alkyl chains.
Column: 0.3 m x 4 mm
LiChrosorb RP-18
Eluent: methanol/water;
composition indicated on the plots

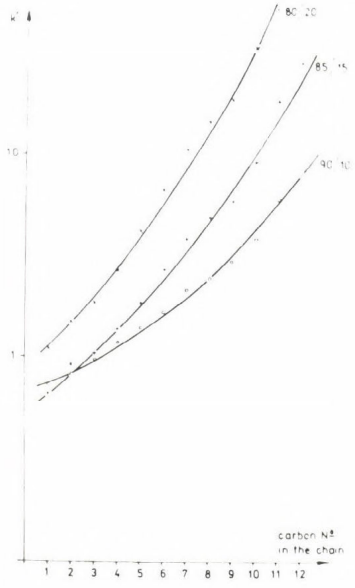

Fig. 7.
Relationship between log k' and the carbon number of the alkyl chains.
Column: 0.3 m x 4 mm
Nucleosil-10-C_8
Eluent: methanol/water;
composition indicated on the plots

tely linear, the plots only serve for informative identification. Within the same laboratory, however, such relationships can be used in the analysis of a great number of samples.

The separation of the diheptyl and di(ethylhexyl) (octyl) phthalates is specially worth mentioning. These components can be distinguished very well by HPLC while they elute together on a packed column in gas chromatography (Figs. 9 and 10).

Figure 11 shows the results of HPLC analysis of plasticizer extracted from the ready-made PVC foil with ether (b) and the original plasticizer (a). The original composition of the plasticizer can be well recognized in the extract: the number of the components increased only in the front part of the chroma-

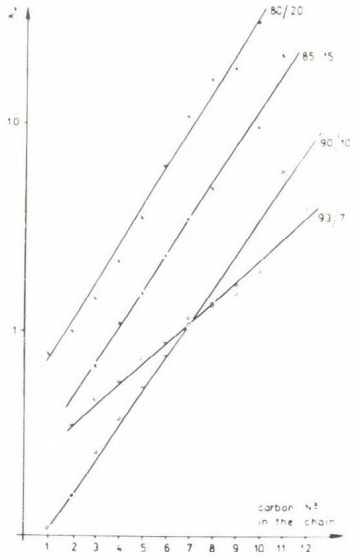

Fig. 8. Relationship between log k' and the carbon number of the alkyl chains.
Column: 0.15 m x 4 mm, LiChrosorb RP-2
Eluent: methanol/water; composition indicated on the plots

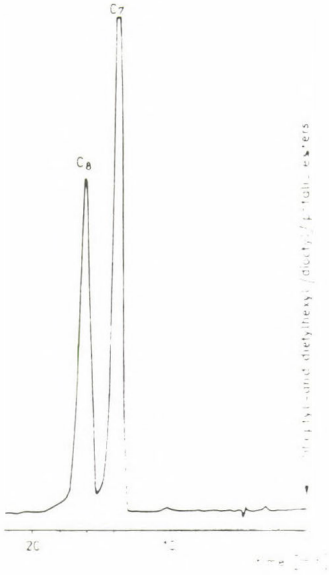

Fig. 9. Separation of diheptyl and dioctyl phthalates.
Conditions: see in Fig. 1

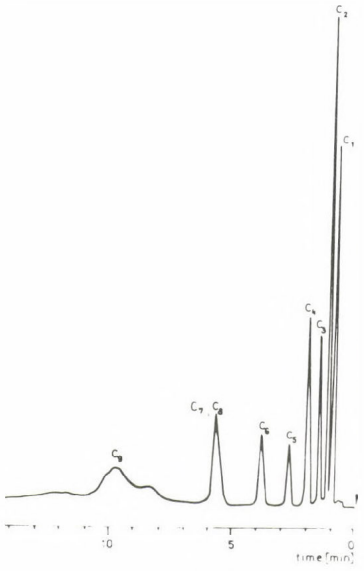

Fig. 10. Separation of phthalate standards by GC. Column: 2 m x 3mm, 10% SE-30 on Chromosorb W. Temperature of injector: 330°C; column temperature: 290°C. Carrier gas: nitrogen, at 30 ml/min. Flame ionization detector

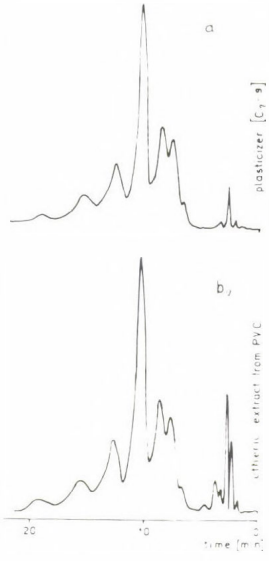

Fig. 11. Separation of plasticizer extracted from a PVC sheet. Conditions: see in Fig. 1

togram; these probably come from the solvent used for the extraction.

With our investigations we have proved that HPLC is suitable for the analysis of a wide range of phthalates; it gives sensitive detection with UV; and provides a better resolution for C_7 and C_8 phthalates.

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HPLC METHOD TO STUDY THE DECOMPOSITION REACTION OF BENOMYL TO CARBENDAZIME

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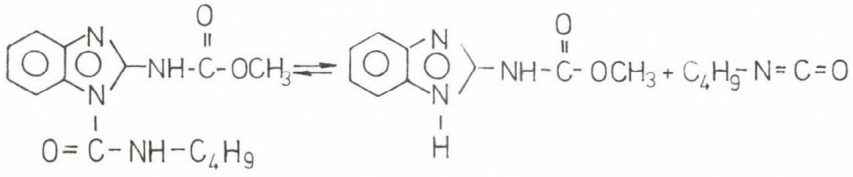
Benomyl (Fundazol 50 WP in Hungary) is a well-known fungicide. The determination of residual benomyl by UV spectrophotometry in soil, waste-water and plant material has been the subject of many reports /1-3/. Methods have also been described for the measurement of benomyl in the form of its first or second degradation product, carbendazime or 2-aminobenzimidazole, by HPLC /4-10/. A number of carbamate pesticides have been separated using HPLC by Sparacino and Hines /11/ as well as by Aten and Bourke /12/. These authors did not take into account the circumstance pointed out by Chiba /13/ namely that benomyl dissolved in organic solvents undergoes a relatively quick decomposition to carbendazime:



benomyl carbendazime n-butyl isocyanate

Using UV spectroscopy, Chiba and Cherniak /14/ carried out kinetic studies of the decomposition and calculated the equilibrium constants. Chiba and Veres /15/ developed an HPLC method separately determine the residue of benomyl and carbendazime: the latter was detected in the form of the propyl isocyanate adduct.

EXPERIMENTAL

The aim of the present work was to find a method to determine the actual carbendazime content in benomyl accounting for the change due to its decomposition in solvents.

Materials and Reagents

Benomyl and carbendazime were the products of Chinoin Pharmaceutical Works, Budapest. Chloroform, methanol, ethanol, trichloroethylene, dimethyl sulfoxide were obtained from Reanal Fine Chemicals, Budapest. UV-grade acetonitrile (Merck) was used as the mobile phase component. Water was distilled from glass.

Apparatus

A Unicam SP 1800 UV spectrophotometer and a Varian Model 5000 Liquid Chromatograph with a Model LC-3 Pye Unicam variable wavelength UV detector were used. The following columns were used: Alltech RP-18 and Hibar LiChrosorb RP-8, both with 10 μm particles. The column dimensions were: 250 x 4 mm.

Following mobile phases were used: 80:20 (v/v) methanol: water, and 85:15 (v/v) acetonitrile: water, at the following flow rates: 1.3, 1.0 and 0.6 ml/min. The UV detector was used at 290, 254 and 245 nm wavelengths.

Sample preparation

(1) 2-3 mg benomyl and 0.2 mg carbendazime were dissolved in 10 ml of acetonitrile (ice-cold or at room temperature). The solution was injected immediately and then in ten-minute intervals.

(2) 10.0 mg of benomyl or 1.0 mg of carbendazime were dissolved in 10 ml of an ice-cold mixture of 1:1 (v/v) trichloroethylene and dimethyl sulfoxide.

RESULTS AND DISCUSSION

HPLC chromatogram

Benomyl and carbendazime were readily separated under the conditions given, however, the results varied as a function of time. When benomyl was injected immediately after its dissolution, its carbendazime content was 4-6% (w/w). When the sample was injected repeatedly, the percentage of carbendazime increased from time to time, in each case. A typical series of chromatograms in shown in Fig. 1.

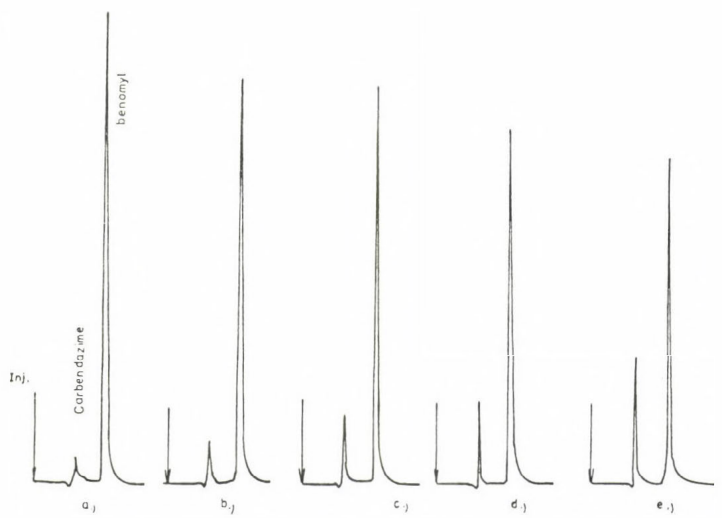


Fig. 1. Analysis of benomyl and its metabolite, carbendazime. Stationary phase: RP-18. 10 µm. Column dimensions: 250 x 4 mm. Mobile phase: 80:20 (v/v) acetonitrile: water. Flow rate: 1.3 ml/min. Detection at 290 nm. (a): Injected 3 min after dissolution: (b): after 10 min; (c): after 17 min; (d): after 24 min; (e): after 67 min

The decomposition of benomyl was studied in different solvents as a function of the temperature and the time elapsed between dissolving and injecting the sample (Fig. 2.)

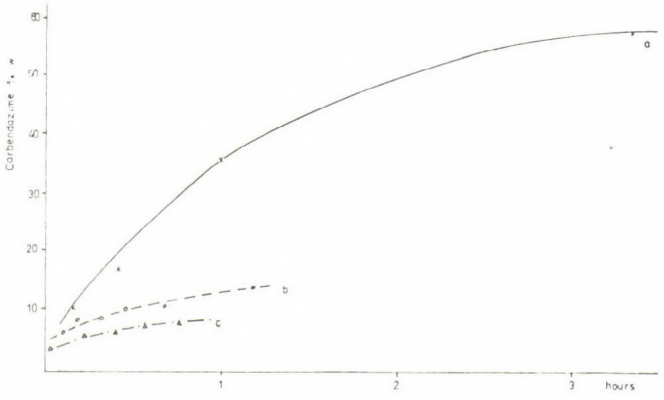


Fig. 2. The rate of degradation of benomyl to carbendazime in different solvents and at different temperatures. (a): 25 °C (room temperature), concentration: 0.155 mg benomyl in 10 ml acetonitrile; (b) 0 °C (ice-water bath), con.: 10.3 mg in 10 ml 1:1 (v/v) trichloro-ethylene:dimethyl sulfoxide; (c): 0 °C, conc.: 2.72 mg in 10 ml acetonitrile. Stationary phase: RP-18, mobile phase: 80:20 (v/v); methanol: water flow rate: 1.0 ml/min: detection at 290 nm

UV spectra

The UV spectra of benomyl and carbendazime were measured in different solvents and at different times after dissolution (Fig. 3).

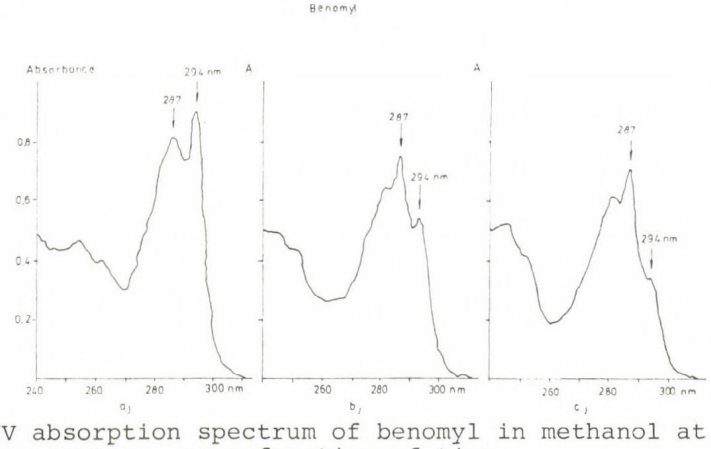


Fig. 3. UV absorption spectrum of benomyl in methanol at room temperature, as a function of time. (a): Immediately after dissolution; (b): One hour later; (c): two hours later

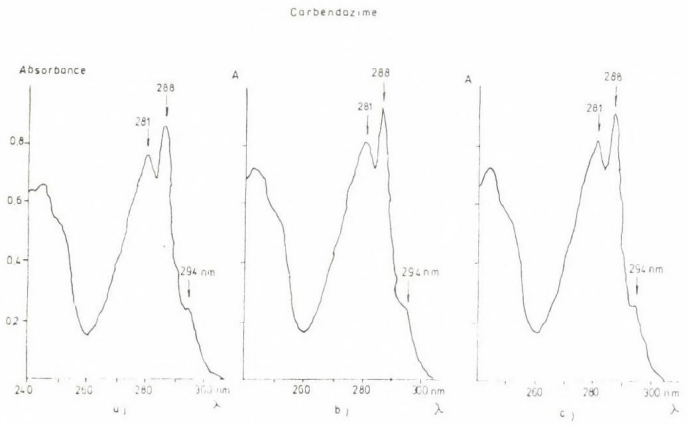


Fig. 4. UV absorption spectrum of carbendazime in methanol at room temperature. (a): Immediately after dissolution; (b): one hour later; (c): two hours later

It can be seen from Figs 3 and 4 that two hours later the spectrum of the benomyl solution has become similar to that of carbendazime.

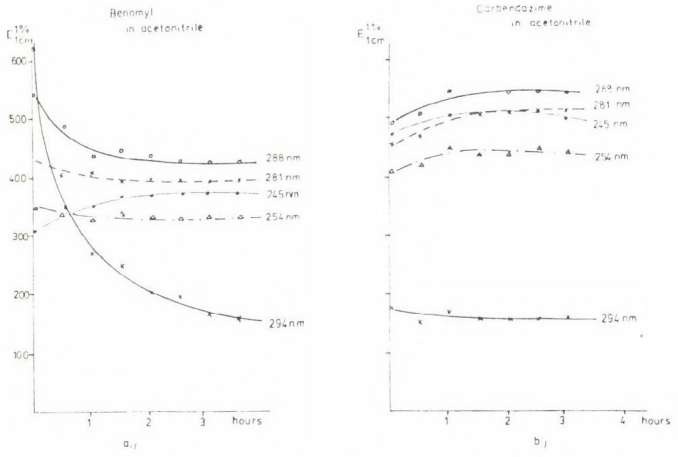


Fig. 5. Time dependence of the specific absorbance coefficients ($E^{1\%}_{1\ cm}$) of solutions of benomyl (a) and carbendazime (b) in acetonitrile on time. $E^{1\%}_{1\ cm} = \frac{A}{c}$ where A is the measured absorbance in the solution and c is the concentration (g/100 ml solution). Path length: 1 cm

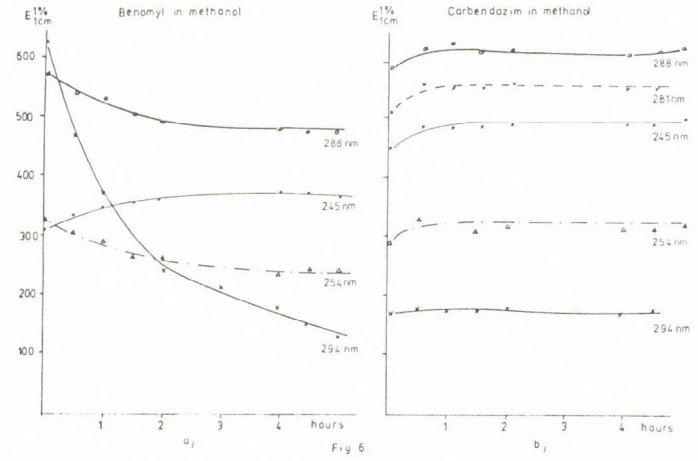


Fig. 6. Time dependence of $E^{1\%}_{1\ cm}$ of a solution of benomyl (a) and carbendazime (b) in methanol

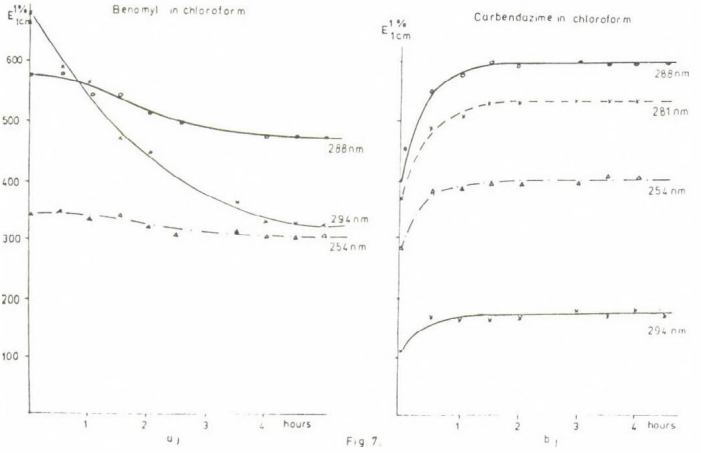


Fig. 7. Time dependence of $E^{1\%}_{1\ cm}$ of a solution of benomyl (a) and carbendazime (b) in chloroform

It can be seen from Figs 5, 6, and 7 that the absorbance of carbendazime increases differently in time in the different solvents. This fact has not yet been mentioned in the literature.

Regarding the different molar and specific absorbances and their time dependence, the carbendazime content of benomyl has been determined with the help of the external standard method. The concentration of carbendazime has been plotted against the time and extrapolated to zero. This value is regarded as the real carbendazime content of benomyl.

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Akadémiai Kiadó
Budapest, 1986

Chromatography '84
H. Kalász and L.S. Ettre (Eds)

HPLC ANALYSIS OF THE REACTION PRODUCTS OF NITROMETHANE WITH FORMALDEHYDE

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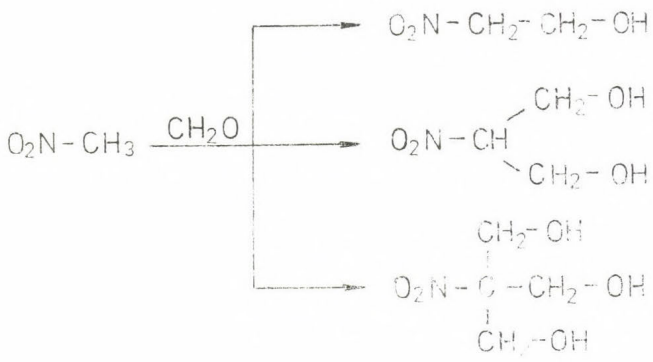
INTRODUCTION

Nitromethane has been known for long time to react with 1-3 moles of formaldehyde leading to hydroxymethylated products:



In 1934, Gorski and Makarov separated the different products by vacuum distillation /1/. No other method has been reported in the literature for the analysis of the reaction mixture.

Two years ago at the 9th Annual Hungarian Symposium on Chromatography, we presented a paper on the analysis of such products by gas chromatography /2/. In the present paper we describe the application of HPLC for the detection and determination of the products and by-products of this reaction.

EXPERIMENTAL

Apparatus

A Liquichrom 2010 (Labor MIM, Budapest) liquid chromatograph equipped with a UV detector was used. Two columns have been found useful: a 250 x 4 mm column packed with 10 µm Chromsil C18 and another column of the same dimensions, packed with 10 µm Chromsil Si. The eluents used were mixtures of water, methanol, dioxan and ethyl acetate in various concentrations for the reversed-phase column and mixtures of ethanol, ethyl acetate and hexane for the silica column. The flow rate of the eluents was 1 ml/min in both cases.

Materials

2-Nitroethanol, 2-nitro-1,3-propanediol and 2-hydroxymethyl-2-nitro-1,3-propanediol were prepared according to Gorski and Makarov /1/. Nitromethane and nitroethane were Fluka products. Nitroethylene was prepared from 2-nitroethanol by heating it with phthalic anhydride. 5-Nitro-1,3-dioxan is a new compound and was synthesized as follows: One gram of o-nitro-1,3-propanediol, one gram of paraformaldehyde and one drop of sulfuric acid were heated on a steam bath for one hour; subsequently the mixture was distilled in vacuum. The product was recrystallized from ethyl acetate, m.p. 37-38 ^{o}C. Elementary analysis: calculated: C:36.1; H: 5.3; N: 10.5. Found: C: 36.2; H: 5.4; N: 10.3. IR: 3000-2800, 1550, 1500, 840 cm^{-1}. ^{13}C-NMR (CCl_4): 93.96 (t) C4, C6; 77.51 (d) C5; 66.36 (t) C2 (ppm).

2-Nitropropanol was prepared analogously to 2-nitroethanol. A solution of methazonic acid in water was obtained by reacting nitromethane with sodium hydroxide at room temperature.

RESULTS AND DISCUSSION

Using the RP-18 column, we were unable to find a suitable eluent effecting the proper separation of 2-nitroethanol and

2- nitro-1,3-propanediol. The sequence of elution when using ethyl acetate-methanol-water were as follows:

2-hydroxymethyl-2-nitro-1,3-propanediol
2-nitroethanol and 2-nitro-1,3-propanediol
2-nitropropanol
nitromethane
nitroethane
nitropropane
methazonic acid

The C18 column can be used with advantage for detecting methazonic acid, which is a dangerous explosive component of the reaction mixtures.

On silica gel, we could separate 2-nitroethanol from 2-nitro-1,3-propanediol with 70:10:20 (v/v) hexane-ethyl acetate-ethanol (90%) used as the eluent. The relative retention times of the compounds were as follows (nitropropane = 1.0):

nitropropane	1.0
nitroethane	1.13
nitromethane	1.28
nitroethylene	1.41
2-nitropropenol	1.63
2-nitopropanol	1.71
5-nitro-1,3-dioxan	1.84
2-nitroethanol	1.93
2-nitro-1,3-propanediol	2.5
2-hydroxymethyl-2-nitro-1,3-propanediol	2.8

The separation of the last two compounds was sometimes unsatisfactory. It appears that the different water content of the eluent was responsible for this.

When analyzing reaction mixtures obtained from the reaction of technical grade nitromethane with formaldehyde, each of the above listed compounds could be detected. Technical grade nitromethane is known to contain nitroethane and the two nitropropanes (1-nitro- and 2-nitropropane). All these compounds react with formaldehyde, but we could identify only 2-nitropropanol of the hydroxymethylated contaminants because of the low concentration of the others.

Nitroethylene and 2-nitropropanol were formed from 2-nitroethanol and 2-nitro-1,3-propanediol, respectively, by water elimination. Due to lack of pure standards, these compounds could not be determined quantitatively in the reaction mixtures (both derivatives are unstable).

5-Nitro-1,3-dioxan is produced from formaldehyde and 2-nitro-1,3-propanediol if the reaction is terminated by acidification before all formaldehyde has been used up.

For quantitative determination, a standard mixture was injected and the results were evaluated by the external standard method.

SUMMARY

The HPLC method proved to be useful for analyzing the mixtures resulting from the reaction of nitromethane with formaldehyde.

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H. Kalász and L.S. Ettre (Eds)

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF 2,4,6-HALOALKYL-1,3,5-TRIAZINES

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**Icechim-Ccmp, Laborator "Solventul"
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ABSTRACT

A fast and simple HPLC method was developed for the separation of 2-alkyl-4,6-bis(trichloromethyl)-1,3,5-triazines and 2,4,6-haloalkyl-1,3,5-triazines.

Procedures for the determination of reversed-phase column performance, using the graphical method of Barber and Carr and the Bristow-Knox algorithm were evaluated.A computer program in BASIC has been developed for the calculations of therelevant chromatographic parameters from the experimental data. The elution order and chromatographic behaviour of nine triazine compounds suggested a structure-retention relationship.

INTRODUCTION

1,3,5-Triazine derivatives are used mainly in agriculture as herbicides, but they also have fungicidal properties and some of them are utilized as pharmaceuticals. Recently, very effective 1,3,5-triazine flame retardants for urethane foams are reported /1/.

Since this chemical class consists of a great number of structurally closely related compounds for which it is difficult to use classical analytical procedures for identification and quantitative assay, chromatographic methods (GC, TLC and HPLC) predominate their determination /2-5/.

High-pressure liquid chromatography (HPLC) has been found to offer a rapid method for the analysis of a large number of 1,3,5-triazines /6-8/. Apparently there is no published information describing HPLC separation of 2,4,6-haloalkyl-1,3,5-triazines or 2-alkyl-1,3,5-triazines.

This paper reports on a HPLC method suitable for a wide range of 2,4,6-haloalkyl-1,3,5-triazines that can be performed with minimum sample preparation. The method has been developed in order to follow the 2,4,6-haloalkyl-1,3,5 triazine synthesis by the acid-catalyzed cyclotrimerization of the corresponding haloalcane nitriles /9/. Moreover, it has been found that chromatographic retention-electronic and steric structure relationships exist for possibly predicting the structure of the 1,3,5-triazines by using retention time, polar substituent (Taft) constants and steric substituent constants, assigned to each substituent group on the triazine ring.

EXPERIMENTAL

A Waters 244 Liquid Chromatograph with a 6000 A high-pressure pump, a Rheodyne 7125 loop injector and a Varian 5020 Liquid Chromatograph (automated VALCO loop injector) with UV detectors operating at a wavelength of 254 nm were used. A Varian 9176 dual channel recorder and a Control Data System CDS 111 L were used. Chromatographic separations were accomplished by using a RP-18 Brownlee cartridge (10 cm x 4 mm i.d.). Experiments concerning kinetic efficiency of the reversed-phase column in the separation of standard triazines have been carried out on the Varian 5020 Liquid Chromatograph equipped with a Micro Pak MCH-10 (Varian) column, 30 cm x 4 mm i.d.

The mobile phase composition was initially adjusted to 90% water and 10% acetonitrile, the polarity of which was adjusted by linear gradient elution for a 20-min period at a flow rate of 1.2 ml/min. The best separation for all compounds and the best compromise between resolution and analysis time were obtained when the linear gradient was adjusted from 55% acetonitrile to 100% acetonitrile.

Table 1. Physical and spectral properties of 2,4,6-haloalkyl-1,3,5-triazines

R_1	R_2	R_3	B.P. °C/mmHg M.P. °C(solv)	UV(MeOH λ_{max},nm( )	IR cm^{-1}	^{1}H-NMR ($CDCl_3$) ,ppm
CH_3	CCl_3	CCl_3	96-97(EtOH)	220 (1925) 280 (945)	1545 1340	7.05 (s,3H)
CCl_3	CCl_3	CCl_3	92-93(EtOH)	220 (2595) 289 (795	1547 1340	-
C_2H_5	CCl_3	CCl_3	160-163/10 33-35	222 (1775) 279 (575)	1529 1545	8.65 (t,3H) 6.8 (q,2H)
$n\text{-}C_3H_7$	CCl_3	CCl_3	175-178/15	221 (1824) 279 (660)	1530 1550	8.9 (t,3H) 8.0 (h,2H) 6.8 (t,2H)
$n\text{-}C_4H_9$	CCl_3	CCl_3	160-163/5	218 (1825 276 (614)	1525 1545	8.50 (s)
$n\text{-}C_{15}H_{31}$	CCl_3	CCl_3	205-207/5	219 (1810 275 (650)	1522 1550	8.72 (s)

R^1	R^2	R^3	Elemental Analysis				
				C %	H %	N %	Cl %
CH_3	CCl_3	CCl_3	calc.	21.84	0.92	12.74	64.49
			found	21.59	0.81	13.02	64.73
CCl_3	CCl_3	CCl_3	calc.	16.64		9.70	73.66
			found	16.59		10.05	73.63
C_2H_5	CCl_3	CCl_3	calc.	24.42	1.45	12.21	61.91
			found	24.31	1.38	12.10	61.75
$n\text{-}C_3H_7$	CCl_3	CCl_3	calc.	26.85	1.97	11.74	59.44
			found	26.69	1.85	11.70	59.32
$n\text{-}C_4H_9$	CCl_3	CCl_3	calc.	29.06	2.44	11.30	57.20
			found	29.15	2.48	11.36	57.28
$n\text{-}C_{15}H_{31}$	CCl_3	CCl_3	calc.	45.63	5.89	7.98	40.49
			found	45.28	5.49	7.80	40.28

The column pressure was 1500 psi. The injection volume varied but was generally 20 microliters.

Acetonitrile was better than methanol for the separation of the compounds analyzed. It was of UV spectro-grade distilled-in glass. Water was treated with kalium permanganate and double-distilled in glass. Solvents were on-line filtered before use.

2,4,6-Haloalkyl-1,3,5-triazines were synthesized by acid-catalyzed cyclotrimerization of the corresponding alcane or haloalcane nitriles and were purified by recrystallization from ethanol or fractional distillation under reduced pressure. Their physical and spectral properties are given in Table 1.

RESULTS AND DISCUSSION

Figs 1 and 2 show the separation of 2-alkyl-4,6-bis(trichloromethyl)-1,3,5-triazines and 2,4,6-haloalkyl-1,3,5-triazines, respectively, on microparticulate reversed-phase column (Brownlee RP-18) using acetonitrile water mobile phase gradient. Table 2 gives relative retention times for the 1,3,5-triazines investigated. The sensitivity limits were in the 80-120 ng range by UV detection at 254 nm.

Several experiments have been caried out for the actual kinetic efficiency determination of the HPLC column (MicroPak MCH-10). The main chromatographic parameters of the standard compound, 2-methyl-4,6-bis(trichloromethyl)-1,3,5-triazine were calculated according to the graphical method described by Barber and Carr and by using the authors' tables /10/. The column performance obtained under these conditions was identical to that computed by the BASIC program developed for a microcomputer MC-18. This program is based on the Bristow-Knox standardized testing procedures which convert the experimental parameters into relevant chromatographic parameters, and is the most popular method for checking how good a HPLC column is /11-12/.

As shown in Table 3, $N_{w\ 0,5}$ can be measured with better accuracy and precision than either N_G or N_K at either equal to 0.5 or 0.1 alpha. $N_{w\ 0,5}$ is given also by the more complex

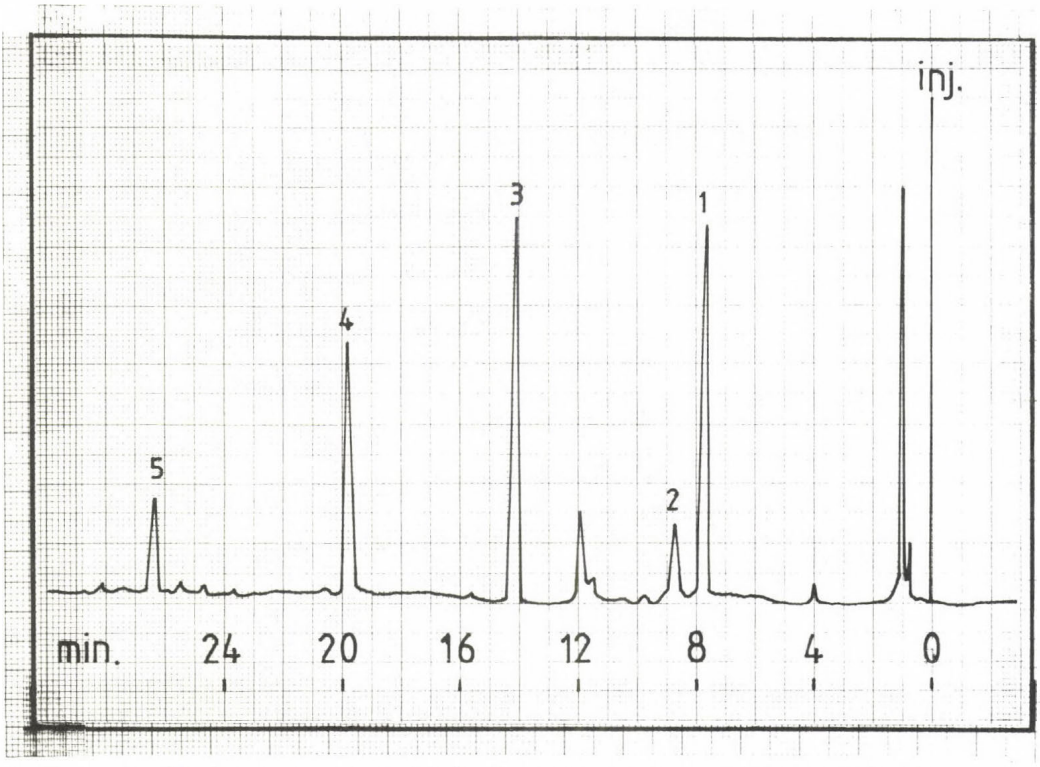


Fig. 1. HPLC separation of 2-alkyl-4,6-bis(trichloromethyl)-1, 3,5-triazines. Waters 244 LC instrument, RP-18 Spheri 5 cartridge Brownlee Labs, acetonitrile-water (55%-100%), flow rate 1.2 ml/min, UV 254 nm, 0.2 AUFS

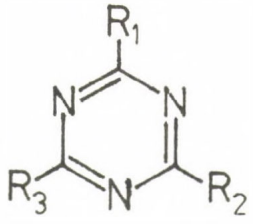
standardized testing program.The main point is to show that if is possible to obtain fundamental chromatographic parameters such as t_R, m_1, $\bar{m}_2$, σ, τ and various estimates of system efficiency such as N_G and N_K with or without automated data acquisition systems or on-line computers.

All the kinetic efficiency experiments and measurements were carried out under isocratic elution after the conversion of the gradient elution conditions to the isocratic mobile phase, keeping constant the k' values.

Retention-structure relationships

The elution order and the chromatographic behaviour of nine alkyl or haloalkyl-1,3,5-triazines suggested a structure-

Table 2. Relative retentions, electronic and steric activity factors of the investigated 1,3,5-triazines.



	R_1	R_2	R_3	t_{Ri}/t_{Ro} [a]	k'	$\Sigma\sigma$ [b]*	ΣE_s [b]
1.	Me	CCl_3	CCl_3	1.00	7.56	5.3	-4.12
2.	Et	CCl_3	CCl_3	1.14 ± 0.02	8.60	5.2	-4.19
3.	n-Pr	CCl_3	CCl_3	1.83 ± 0.01	13.71	5.185	-4.48
4.	n-Bu	CCl_3	CCl_3	2.44 ± 0.03	19.88	5.17	-4.51
5.	$n\text{-}C_{15}H_{31}$	CCl_3	CCl_3	3.40 ± 0.04	25.26	5.21 [d]	
6.	CCl_3	CCl_3	CCl_3	1.52 ± 0.02	11.19	7.95	-6.18
7.	$ClCH_2CH_2$	CCl_3	CCl_3	1.31 ± 0.01	10.27	5.685	-5.02
8.	$ClCH_2CH_2$	$ClCH_2CH_2$	CCl_3	1.27 ± 0.02	9.89	3.42	-3.86
9. [c]	Cl_2CH	Cl_2CH	Cl_2CH	1.13 ± 0.03	8.25	5.82	-4.62

[a] gradient elution from 55% acetonitrile to 100% acetonitrile. Relative retention times ± standard deviation

[b] taken from reference /13/

[c] Synthesized by the chlorination of 2,4,6-trimethyl-1,3,5-triazine

[d] calculated from the data given in reference /13/ (additivity rules). No data in the literature for E_s of $n\text{-}C_{15}H_{31}$ group.

Table 3. HPLC column (MicroPak MCH-10) performance determined by the graphical method of Barber and Carr

Chromatographic parameter	α = 0.5		α = 0.1	
		s % [a]		s % [a]
t_p	321.7647	0.53	321.7647	0.50
W_α	11.7647	3.10	23.5294	2.90
A/B-1	0.1122	4.20	0.1860	3.90
t_r	319.1120	0.74	319.2113	0.71
σ	4.2186	3.04	4.6374	3.00
τ	3.5858	5.10	3.2462	4.70
m_1	322.6978	0.48	322.4575	0.47
$\bar{m}_2$	30.6544	3.70	32.0435	0.30
$N_{w\ 0.5}$	4144	0.75		
N_K	3377	9.76	3231	8.31
N_G	5722	6.30	4738	5.91

[a] relative standard deviation from 10 independent determinations;

N - number of theoretical plates;

t_p - time of appearance of the peak maximum;

t_r - time at the peak apex;

W_α - peak width at ;

$N_{w\ 0.5}$ is calculated by the eq. $N_{w\ 0.5} = 5.54\ (t_p/W_{0.5})^2$;

τ is the time constant of the exponential peak modifier;

σ is the standard deviation of the Gaussian profile;

$N_K = t_p/\bar{m}_2$ where $\bar{m}_2$ is the variance (second central moment);

$N_G = t_r^2/\sigma^2$;

m_1 is the first statistical moment (center of gravity);

$\bar{m}_2 = t_r + \tau$;

$m_2 = \sigma^2 + \tau^2$

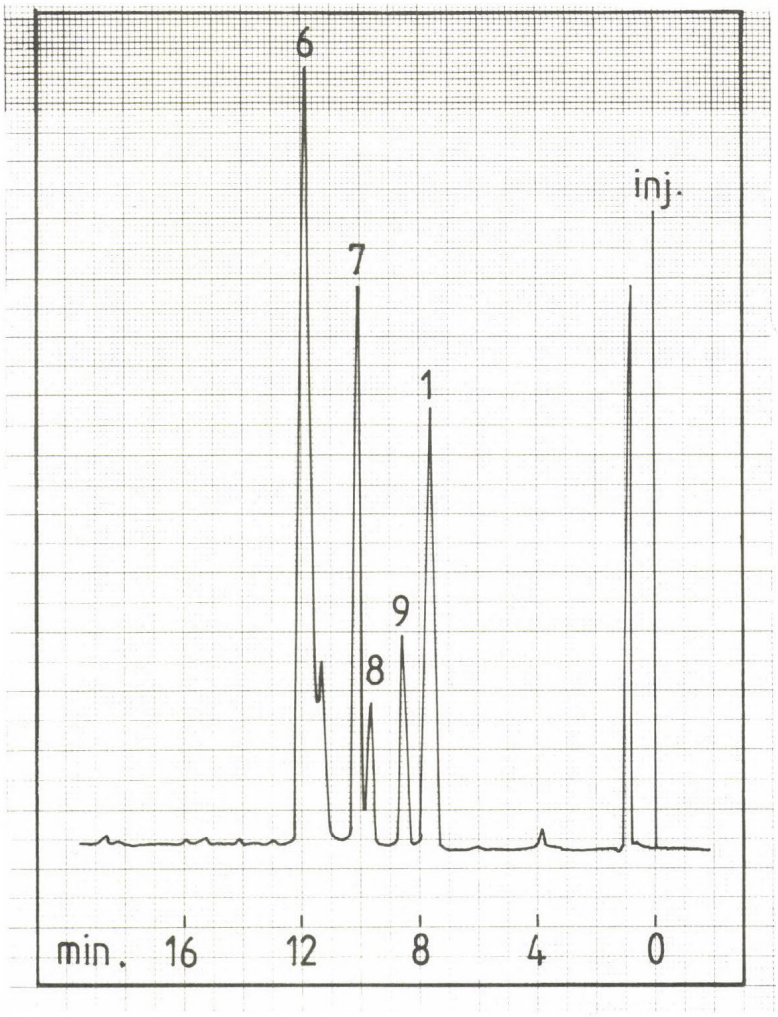


Fig. 2. HPLC separation of 2,4,6-haloalkyl-1,3,5-triazines. Waters 244 LC instrument, RP-18 Spheri 5 cartridge Brownlee Labs, acetonitrile-water (55%-100%), flow rate 1.2 ml/min, UV 254 nm, 0.2 AUFS

retention relationship. Thus, retention could be used to predict qualitative behaviour.

Relating analogously to the Taft equation /13/ a multiple linear regression equation can be deducted relating the relative retention, electronic effect of the substituents on retention and the steric effect on retention:

$$\log \frac{t_{Ri}}{t_{Ro}} = a_o + a_1 \Sigma\sigma^* + a_2 \Sigma E_s \qquad (1)$$

where $\Sigma\sigma^*$ is the electronic activity factor and represents the ability of the substituted group to attract or repel electrons by combination of inductive and mesomeric effects. It is calculated as the sum of the polar substituent constants taken from literature /13/. E_s is the sum of steric substituent constants.

Thus, the a_1 and a_2 parameters can be interpreted as a measure of the electronic and steric sensitivity, respectively, of the particular 1,3,5-triazine series for the resolution on the specified chromatographic column under the stated elution conditions. t_{Ri} and t_{Ro} are the retention times of the substituted and reference 1,3,5-triazines. As the reference 1,3,5-triazine, 2-methyl-4,6-bis (trichloromethyl)-1,3,5-triazine was selected.

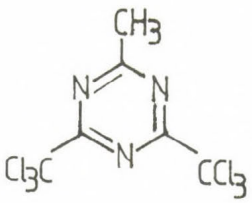
The obtained coefficients of eq. for the 2-alkyl-4,6-bis (trichloromethyl)-1,3,5-triazines series (compounds 1 - 5 in Table 2) and 2,4,6-haloalkyl-1,3,5-triazines series (compounds 6 - 9 in Table 2) are given in Table 4.

For the 2-alkyl-4,6-bis(trichloromethyl)-1,3,5-triazine series a similar equation was deducted by the correlation of the logarithm of relative retention with the electronic activity factor

$$\log \frac{t_{Ri}}{t_{Ro}} = -2.556\Sigma\sigma^* + 13.525 \quad (r = 0.910) \qquad (2)$$

Table 4. Structure-retention relationship for haloalkyl-1,3,5-triazines

Triazines	a_o	a_1	a_2	r
2-Alkyl-4,6-bis (trichloromethyl)-1,3,5-triazines (compounds 1 - 4)	4.380	-1.210	-0.491	0.947
2,4,6-Haloalkyl-1,3,5-triazines (compounds 1, 6 - 9)	-0.345	-0.0753	-0.180	0.976



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220PRINT" DIFFUSIVITY M2/S";
230INPUTF
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260PRINT"CHART SPEED MM/S";
270INPUTS
280PRINT"ELUENT DISTANCE MM, SOLUTE DISTANCE MM";
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350INPUTI
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490PRINT"PARTICLE DIA      :";D;" UM       REDUCED LENGTH :";D1
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520T1=T1/S
530W1=W1/S
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700Q=Q*1E-6/60
710V=V*1E-3
720N=5.54*T1*T1/(W1*W1)
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740U=L/T0
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760K=U*V*L/P
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781PRINT"=============================================================================
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805H1=H*1.E6
810H2=H/D
820PRINT"HETP             : "H1" UM      REDUCED H            : "H2
830U1=U*1000
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850D2=D*D/K
860PRINT"PERMEABILITY     : "K" M2       RESISTANCE           : "D2
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880H1=H*H/K
890PRINT"PERF INDEX       : "K1" /BAR S SEPARATION IMPEDANCE: "H1
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921PRINT
922PRINT"                 INFINITE DIAMETER MODE NOT POSSIBLE"
923PRINT
924PRINT"KNOX-PARCHER: "B1
930GOTO960
940B1=B*B/(L*D)
952PRINT
953PRINT"                 INFINITE DIAMETER MODE POSSIBLE"
954PRINT
955PRINT"KNOX-PARCHER RATIO: "B1
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CHART SPEED MM/S
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:10.2,54.7,
SOLUTE WIDTH AT HALF HEIGHT MM
:2
PRESSURE BAR, FLOWRATE ML/MIN
:32,1,
INJECTION VOL UL
:10E-03,
                    TEST CONDITIONS AND INPUT DATA
==========================================================================

VISC              : 5.79999E-04 N S/M2
DIFF              : 7.89999E-10 M2/S
COL LENGTH        : 3.00000E 02 MM      COL BORE          : 4.00000E 00 MM
PARTICLE DIA      : 1.00000E 01 UM      REDUCED LENGTH    : 3.00000E 04
ELUENT TIME       : 6.00000E 01 S       SOLUTE TIME       : 3.21764E 02 S
SOLUTE BASE WIDTH: 2.00000E 01 S        CHART SPEED       : 1.69999E-01 MM/S
PRESSURE DROP     : 3.20000E 01 BAR     FLOW RATE         : 1.66666E-02ML/S
INJECTION VOL     : 9.99999E-03 UL
SOLUTE PEAK       : 3.33333E-01 ML
```

```
                    CHROMATOGRAPHIC PARAMETERS
 ================================================================================

 PLATE NUMBER     :  4.14404E 03
 HETP             :  7.23930E 01 UM      REDUCED H              :  7.23930E 00
 LINEAR VELOCITY  :  4.99999E 00 MM/S    REDUCED VELOCITY       :  6.32910E 01
 PERMEABILITY     :  2.71874E-13 M2      RESISTANCE             :  3.67816E 02
 PERF INDEX       :  1.66786E 03 /BAR S  SEPARATION IMPEDANCE   :  1.92763E 04
 KNOX-PARCHER     : 5.33333E 00

                  INFINITE DIAMETER MODE NOT POSSIBLE

 KNOX-PARCHER:  5.33333E 00
 TOTAL POROSITY     :  2.65393E-01
 COLUMN CAP. FACTOR:  4.36274E 00          RETENTION RATIO    :  1.86471E-01

  STOP AT 9998

 READY
 920
```

CONCLUSION

No pretension to theoretical rigor has been made in this study since the objective was to develop methodology which would allow quantitation and reasonable qualitative prediction of a variety of substituted 1,3,5-triazines, and since a topological method is to be investigated at present. The identification of a particular compound by this method is very useful in the absence of a mass spectrometer.

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